International Symposium
Genes and Chromosomes
Structure and Function

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International Symposium Genes and Chromosomes Structure and Function

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GENES AND CHROMOSOMES
STRUCTURE AND FUNCTION

Held at

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November 30 – December 4, 1964

Edited by
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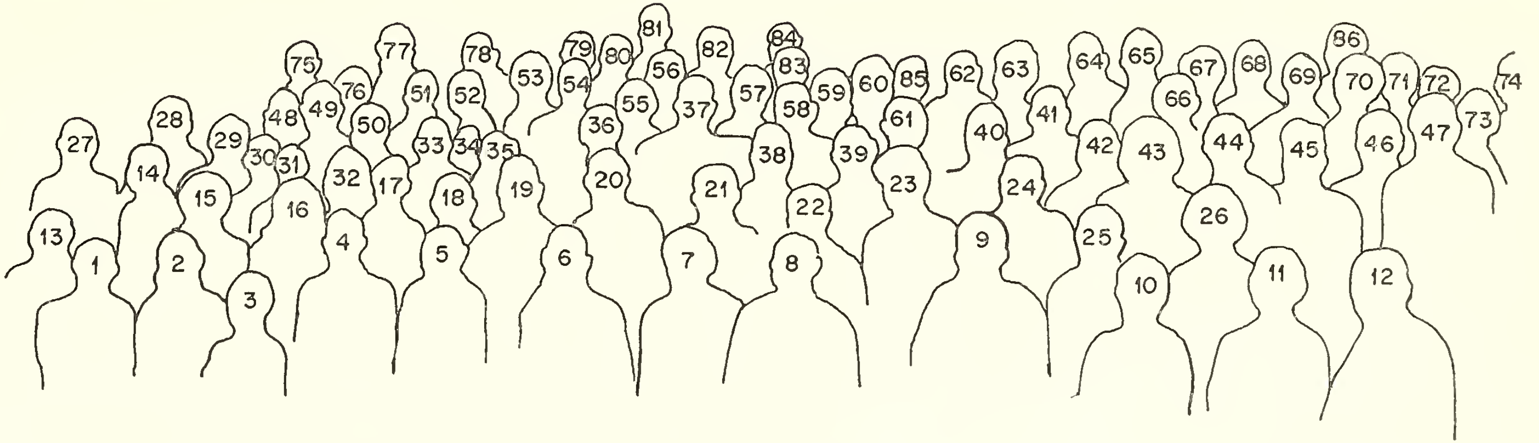
and Dr. Rhoda F. Grell

With the cooperation of Dr. Ruby Marie Valencia.



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FOREWORD

The organization of high-level symposia in several of the main centers of biological research in Latin America during the past 4 or 5 years has been an important stimulus to those research groups which have been making a big effort for many years to develop modern biology by forming a nucleus of young people capable of teaching and doing original research. To do this in cytology and genetics in Argentina, it has been necessary to start with very little or nothing in the way of space, modern equipment, or money, in the midst of economic and political instability. It has been possible to continue our efforts due to the understanding and support of a small but optimistic and very tenacious group of leaders, such as Dr. Rolando García, Dean of the School of Science, and Professor Bernardo Houssay, President of the National Research Council, who are conscious of the importance of these endeavors. They believe that the formation of a large elité of scientists and technically trained people and the development, through science, of a progressive mind in the general population can play a very important role in the attainment of full maturity in a country.

It was problems of this general nature that led us, in 1958, to ask Dr. Hollaender to come and visit us to assess our particular situation and arrange some kind of scientific help. This symposium could be regarded as the culmination of this relationship.

It should be pointed out that these Latin American symposia cannot be compared to the typical symposium held in the United States, for example, in which a limited number of selected scientists meet and discuss a limited topic in a thorough and profound manner. Symposia such as this one must have an additional purpose, which takes priority over the exhaustive treatment of a theme. It is the making of a unique opportunity for Latin American students and scientists, especially the younger ones, to get to know and be stimulated by top scientists from advanced countries, as well as for the latter to become aware of the Latin Americans and their problems. To accomplish this aim, the doors must be left open to all local and neighboring scientists, students, and educators who wish to attend and can manage to do so.

Surely all members of the symposium will agree that participation in meetings of this kind implies much more responsibility than just to read a paper and/or take part in discussions during the conference. To be really successful the symposium should leave something concrete behind, such as permanent personal contacts, which in time can result in the betterment of specific research groups. The tremendous influence that a very small but well-placed personal effort can have among our young and well-inspired scientists has been demonstrated here in Buenos Aires. We would like to mention specifically the groups oriented 3 years ago, by two outstanding French investigators, Dr. Elie Wollman and Dr. Jerome Lejeune. Both came for a very short time, but thanks to their good will and continued interest, we have today in our school two groups doing research and teaching in bacterial genetics and human cytogenetics, at a level such that their work has attained international recognition. Symposia such as this one could be the foundation for the multiplication of these examples. Each person coming to a symposium from an advanced research institution should feel it his obligation, during his short visit, not only to make a firm personal contact with the local scientists working in his own field, but also to inform himself as thoroughly as possible concerning the organization and administration of the institutions of research and education, the problems that are faced, the kind of help which is needed, etc. The truth is that the problems we face are so different from those encountered in scientifically advanced countries that it is hard for an outsider to comprehend them, but an individual who has made the effort and understands the situation is in a position to offer his help in the most constructive way, not only through his personal research collaboration but also by trying to obtain a more realistic canalization of international aid. For example, the kind of help which is most needed is the kind which will enable and encourage our young scientists to remain here. All too often outside help results in the loss of the scientists from the country.

It is certain that the human material available to science in Argentina is excellent. We have found an unusual degree of intelligence, formal education, interest, and will power among the young people who have come to join us. They compare favorably with the best of any country, and are, in fact, perhaps superior to the average found in advanced countries, since the obstacles met on the way up allow only the best to arrive. A person of mediocre intelligence or poor insight or little will power might manage to produce in an institution where he has all material facilities and the benefit of the advice and stimulation of numerous colleagues. In a place lacking all this, he could produce nothing and would probably fall by the wayside. In view of these considerations, a research work produced by a scientist in a Latin American country usually cannot and should not be judged by the same yardstick as research produced in an advanced institution in the United States, for example. It may have taken quite a long time to be done, it may involve methods no longer in use elsewhere, etc., but the human effort that went into it was probably exceptionally great.

Another very important consequence of international scientific meetings can be the awakening, within the government of the host country, of an awareness of the importance of the development of modern science. Officials who might ignore the pleas of local scientists are likely to take note of an illustrious group of foreign investigators and through them begin to appreciate the role of science in a modern country, eventually arriving at the conviction that at this moment in history, science must be taken into consideration in government planning at all levels.

It is our hope that in the course of time this symposium will be found to be beneficial to Argentina in the ways we have discussed here. Certainly it was stimulating, interesting, and in every way pleasing to us while it lasted.

Juan I. Valencia
Ruby M. Valencia

TABLE OF CONTENTS

Page

Introduction
ALEXANDER HOLLAENDER XIII

Genes: Structure and Function

Gene-Protein Relationships
GEORGE STREISINGER 1

Genetic Control of the pH 7.5 Esterase in Maize
DREW SCHWARTZ 9

Gene Differentiation
M. DEMEREC 15

Neurospora Mutants Deficient in Aspartic Transcarbamylase
J. L. REISSIG, A. S. ISSALY, M. NAZARIO, and A. J. JOBBÁGY 21

Impaired Complementation Between Nonallelic Mutations in *Neurospora*
FREDERICK J. DE SERRES 33

Genetic Aspects of Colicinogeny
R. NAGEL DE ZWAIG and D. N. ANTÓN 53

Chromosome Structure and Replication

The Bacteriophage Chromosome
R. S. EDGAR 67

Fine Structure of Lampbrush Chromosomes
O. L. MILLER, JR. 79

Chromosome Replication
W. J. PEACOCK 101

Fine Structure of Meiotic Chromosomes
J. ROBERTO SOTELO and RODOLFO WETTSTEIN 133

Chromosome Breakage and Reunion

On the Chemistry of Chromosome Continuity
SHELDON WOLFF 155

Modification of Breakage and Reunion in Relation to Lethal Damage
CARMEN B. DE LOZZIO and JUAN I. VALENCIA 181

Chromosome Behavior

Chromosome Pairing, Crossing-Over, and Segregation in *Drosophila melanogaster*
RHODA F. GRELL 215

The Meiotic Mechanics of Ring Chromosomes in Female *Drosophila melanogaster*
L. SANDLER 243

Page

Chromosome Function at the Supragenic Level
D. L. LINDSLEY 275

Chromosome Metabolism

Chromosome Functions in Specific Regions
H. J. BECKER 293

Chromosomal Differentiation
CRODOWALDO PAVAN 309

The Nucleolus and the Synthesis of Ribosomes
ROBERT P. PERRY 325

Symposium on Structure and Function of Genes and Chromosomes: A Summation
NORMAN H. GILES 341

Introduction[1]

ALEXANDER HOLLAENDER, *Biology Division, Oak Ridge National Laboratory,*[2] *Oak Ridge, Tennessee*

The great progress during the last decade in the field of molecular genetics has come largely from experimentation with microorganisms. During the same period there has been considerable progress in the field of chromosomal cytology and genetics. It seems quite likely that the next years will see the molecular biologists turning to problems on the chromosomal level and increasing use of the techniques and concepts of molecular biology by chromosomal biologists. For this reason, it seems timely to bring together investigators from the two disciplines at a symposium that will encourage them to extend their thinking into this area of common interest.

This Symposium is the product of the close ties between Dr. Juan I. Valencia of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and Dr. Ruby Marie Valencia of the Comision Nacional de Energia Atomica on one hand, and the geneticists of the Biology Division, Oak Ridge National Laboratory, on the other. Specifically it grew out of discussions between the Drs. Valencia, Dr. D. L. Lindsley, and Dr. Sheldon Wolff in Oak Ridge during the spring of 1963. These four formed the organizing committee.

The symposium was opened by Dr. B. Houssay, President of the Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina, who gave us excellent insight into the importance of science in the development of Argentina. Then Dr. R. V. Garcia, Dean of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, gave a very interesting discussion of education and the developing of new ideas in the educational life of the Republic of Argentina. Finally I spoke about the background for these symposia. This is the fourth Latin American conference in basic biology organized jointly by biologists from Latin American laboratories and the Biology Division of the Oak Ridge National Laboratory. These conferences have become extremely important in bringing scientists from the United States and Europe to Latin America for discussions with their colleagues there of new develop-

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[1] Presented at the International Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30–December 4, 1964.

[2] Operated by the Union Carbide Corporation for the U.S. Atomic Energy Commission.

ments in modern biology, especially in the areas where important contributions have been made in Latin American laboratories. They have also served to focus renewed attention on the long and significant history of the host countries and their cultural background. Their accomplishments in the fields of literature, music, and more recently in the visual arts have made a marked impression on the world as a whole.

The symposium was successful not only from a purely scientific point of view, but also from a personal point of view, and was especially successful in that it brought many graduate students, not only from Argentina but also from neighboring countries, into the symposium and into the discussions.

It is hoped that these proceedings will be published soon so that they become available to many investigators all over the world and especially point out the important progress in a quickly developing field which is taking place in Latin America.

Special thanks should be given to the Argentine government, to the United States government agencies and foreign governments who have generously supported this symposium, and to the National Cancer Institute who arranged for this publication.

INTRODUCCION [1] (Prefacio)

El gran progreso durante la última década en el campo de la genética molecular ha venido en gran parte de la experimentación con microorganismos. Durante el mismo período considerable progreso ha tenido lugar en el campo de la genética y la citología cromosomal. Parece muy probable que en los próximos años nosotros veremos a los biólogos moleculares enfocar en problemas al nivel cromosomal y un aumento en el uso de técnicas y conceptos de la biología molecular de los biólogos cromosomales. Por lo tanto parece muy apropiado de reunir en simposio a investigadores de ambas disciplinas que alentarán a ellos a extender sus pensamientos a esta área de común interés.

Este simposio es el producto de las íntimas relaciones entre el Dr. Juan I. Valencia de la Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, el Dr. Ruby Marie Valencia de la Comisión de la Energía Atómica y los geneticistas de la División de Biología del Oak Ridge National Laboratory.[2] Especificamente es el resultado de las discusiones entre los Drs. Valencia, el Dr. D. L. Lindsley, y el Dr. Sheldon Wolff, en Oak Ridge, durante la Primavera de 1963. Estos cuatro formaron el comité de organización.

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[1] Presentado en el Simposio Internacional sobre Genes y Cromosomas—Estructura y Función, Buenos Aires. Argentina, Noviembre 30-Diciembre 4, 1964.

[2] Operada por la Union Carbide Corporation para la Comisión de la Energía Atómica de los Estados Unidos.

El simposio fue abierto por el Dr. B. Houssay, Presidente del Consejo Nacional de Investigaciones Científicas y Técnicas de la Republica Argentina, que nos dió una excelente presentación de la importancia de la ciencia en el desarollo de la Argentina. Entonces, el Dr. R. V. García, Decano de la Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires, dió una muy interesante discusión sobre educación y el desarollo de nuevas ideas sobre la vida educacional en la República Argentina. Finalmente, yo hablé sobre los antecedentes para estos simposios. Esta es la quarta conferencia Latino-Americana en biología básica organizada conjuntamente por biólogos de laboratorios de Latino-América y la División de Biología del Oak Ridge National Laboratory. Estas conferencias han llegada a ser muy importantes para traer científicos de los Estados Unidas y Europa a Latino-América para discutir con sus colegas allí, los nuevos adelantos en biología moderna, especialmente en el área donde las contribuciones han tenido lugar en laboratorios de América-Latina. Ellas tambien han servido para llamar renovada atención de la larga y significante historia de los paises huéspedes y de sus antecentes culturales. Sus logros en los campos de la literatura, música y más recientemente en las artes visuales han hecho una impresión en todo el mundo.

El simposio fue un éxito no solo del punto de vista científico sino también del punto de vista personal, y fué especialmente un éxito porque trajo muchos estudiantes graduados, no solo de la Argentina sino también de los paises vicinos, al simposio y a las discusiones.

Es la esperanza que las actas de esta conferencia serán publicadas pronto, de tal manera que serán accesibles a muchos investigadores de todo el mundo y especialmente hacer notar el importante progreso que ha tenido lugar en Latino-América en este campo de rápidos adelantos.

Agradecimientos especiales tienen que darse al gobierno Argentino, a las agencias del gobierno de los Estados Unidos y de los Gobiernos extranjeros que generosamente han asistido este simposio, y al National Cancer Institute que ha hecho los arreglos para esta publicación.

Genes: Structure and Function

Gene-Protein Relationships [1,]

GEORGE STREISINGER, *Institute of Molecular Biology, University of Oregon, Eugene, Oregon*

THE one gene-one enzyme hypothesis (*1*) initiated an era of extremely fruitful studies concerning gene-protein relationships, studies that have led to a continuing evolution and clarification of that hypothesis.

In this paper I will summarize briefly some current results, predominantly of others, that serve to define precisely the relationship between a gene and a product protein.

The studies of Watson and Crick (*2*), Benzer (*3*), Sanger and Tuppy (*4*), and Ingram (*5*) suggest that the gene may be defined as that region of the deoxyribonucleic acid (DNA) molecule specifying the primary structure of a single polypeptide chain. A very appealing and simple hypothesis, to account for the general way in which the structure of a polypeptide chain could be related to the structure of a gene, was first enunciated by Crick (*6*) who suggested that the sequence of bases in the DNA molecule specifies the sequence of amino acids in a product polypeptide chain and that the two sequences are colinear. I will very briefly describe two sets of experiments that have recently confirmed the colinearity hypothesis.

The general nature of the genetic code, *i.e.*, the way in which a certain sequence of bases in the DNA can specify a certain amino acid in the product protein chain, has again been clarified through experiments of Crick *et al.* (*7*). I shall briefly describe these experiments and some recent ones from my own laboratory concerning this problem.

COLINEARITY HYPOTHESIS

Yanofsky *et al.* (*8*) mapped the sites of several mutations within that region of the DNA molecule which specifies the structure of one of the two polypeptide chains constituting the enzyme, tryptophan synthetase, of *Escherichia coli*. Yanofsky also determined the position of the changed amino acid within the polypeptide chain of each of the mutant strains. The map in text-figure 1 summarizes his results. Clearly, the sequence of

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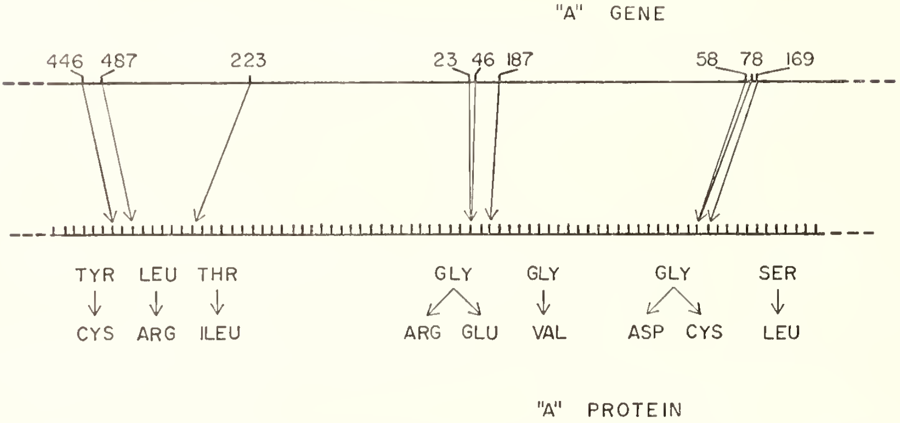
[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 5, 1964.

[2] Research supported by grant 465-GB2261 from the National Science Foundation.

changed amino acids within the polypeptide chain is the same as the sequence of mutations that gave rise to the changed amino acids, and the genetic distances between the sites of mutation are proportional to the distances along the polypeptide chain between altered amino acids.



Text-figure 1.—The relative positions of mutations in the A gene controlling the structure of tryptophan synthetase and the relative positions of changed amino acids in the A chain of tryptophan synthetase. The numbers *above* the line symbolizing the A gene represent different mutations and the distances separating the mutations are proportional to the genetic distances observed by Yanofsky (*8*). Each *vertical* line represents one amino acid. Redrawn from the results of Yanofsky *et al.* (*8*).

Brenner *et al.* (*9*) used an entirely different system for examining colinearity. They used a kind of mutation in phage T4 that has been named "amber." In an amber mutant strain, translation of the genetic message seems to be terminated at the site of the mutation (*10*). If the amber mutant strain is grown on certain special host bacteria that may be designated permissive, translation does not terminate, but instead a normal, or near normal phenotype is restored.

Amber mutations have been found in a variety of different genes in phage T4, including the gene responsible for the head protein, a protein constituting about half of all the proteins synthesized during the latter half of the growth cycle of phage.

Bacteria infected with phage T4 carrying a head amber mutation would be expected to synthesize a polypeptide chain that would terminate at the site of the mutation. Brenner found that the lengths of the head polypeptide fragments produced by various head amber mutants were correlated with the genetic location of the mutations. Mutations near one end of the head gene produced short fragments, while the fragment length increased the farther away the sites of mutation were located from this end.

As had been predicted by Crick, the sequence of bases in DNA and the sequence of amino acids in a product protein are colinear.

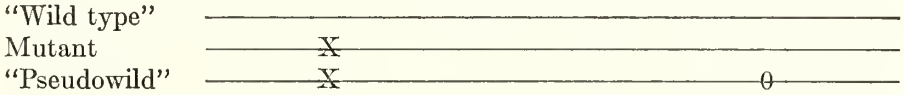
GENERAL NATURE OF THE GENETIC CODE

There have been a number of hypotheses to explain the way in which groups of bases in the DNA may code for particular amino acids (*11–13*). The problem has finally been solved during the last few years by the experiments of Crick and his coworkers (*7*), who used mutations induced by the acridine-dye proflavine for their experiments.

The mutagen proflavine differs in its action from a mutagenic base analogue such as 5-bromouracil in two ways: 1) Mutations induced by proflavine are not induced to revert by any mutagenic base analogue, and 2) proflavine does not induce nonlethal mutations in such essential structures of the phage as the phage head. Because of these features of proflavine mutagenesis, and because of other types of results, Brenner *et al.* (*14*) suggested that proflavine-induced mutations involve additions or deletions of single base pairs.

Proflavine efficiently induces mutations in the r_{II} region of phage T4 (*15*). Mutations in the r_{II} region have been studied exhaustively by Benzer (*3*). Phage strains carrying r_{II} mutations differ from the wild-type strain in two respects: They produce altered colonies (plaques) on the bacterial strain B, and they cannot grow on the bacterial strain K. The function controlled by the r_{II} region is not essential for growth on the bacterial strain B since r_{II} mutants in which sections of the r_{II} region have been deleted grow normally on this strain.

Crick observed that spontaneous (or proflavine-induced) "reversions" of proflavine-induced r_{II} mutations resulted in only a partial restoration of the wild-type phenotype: Revertants grew on K bacteria but often still produced altered plaques on B bacteria. Genetic tests showed that these reversions to a "pseudowild" phenotype were not caused by a reversal of the original change but rather by nearby "suppressor" mutations which, when extracted by means of genetic crosses, were r_{II} mutations as well. A series of r_{II} mutations isolated as suppressors of a particular mutation did not suppress each other, but a series of new suppressor mutations could be isolated that suppressed any particular suppressor mutation (*7*).



These and other experiments can be summarized by stating that within a short region of the genome a group of mutants can be separated into two subsets. In a double mutant strain, two mutations belonging to different subsets give rise to a pseudowild-type phenotype, *i.e.*, they suppress one another, while two mutations belonging to the same subset do not give rise to a pseudowild-type phenotype but rather to a mutant phenotype, *i.e.*, they do not suppress one another.

From these results Crick assumed: 1) That one of the two subsets contains mutations caused by an addition of a base pair while the other subset contains mutations caused by a deletion of a base pair, and 2) that a sequence of bases is translated group by group, *e.g.*, triplet by triplet, into a sequence of amino acids starting from a defined base at the beginning of the sequence (*7*).

In a single addition or deletion mutation, the groups of bases read would differ from those in the wild type from the point of mutation on to the end of the gene. In a deletion mutation followed by an addition mutation, the groups of bases read would differ only within the (short) region included between the two mutations.

Consider, for instance, the short "message:"

. . . CAT ACT GAT TAG . . .

The deletion of a single base, at the beginning of the message, results in:

. . . ↑ATA CTG ATT AG. . . .

but a deletion, followed by an addition, results in the restoration of the message from the point of mutation on:

. . . ↑ATA CT$\overset{\downarrow}{\text{A}}$ GAT TAG . . .

The hypothesis predicts that if the code is a triplet code (or a code involving multiples of a triplet) three mutations of the same subset, when present in a single short section of the genome, would result in a wild or pseudowild phenotype. This result was in fact obtained by Crick (*7*).

In my laboratory we have been examining a number of predictions of Crick's hypothesis regarding the nature of proflavine-induced mutations and the genetic code, using mutations that affect the lysozyme of phage T4.

Phage T4 forms plaques when plated on ordinary media with sensitive bacteria. When petri plates with plaques are exposed to chloroform vapors, halos form around the plaques due to phage lysozyme, which diffuses away from the plaques and digests the cell walls of the chloroform-sensitized bacteria.

When mutant strains that produce temperature-sensitive lysozyme are plated at elevated temperatures, they form plaques having smaller halos or lacking halos (*16*). Mutant strains that have part or all of the lysozyme region deleted form no plaques on ordinary media, but do form plaques when plated on properly adjusted media supplemented with egg-white lysozyme (*17*).

Under the coding hypothesis proposed by Crick, proflavine-induced lysozyme mutations are expected to produce no active lysozyme, and thus to produce no plaques under ordinary conditions, since the lysozyme molecule will have an altered sequence of amino acids from the point of the mutation on to the end of the molecule. An appreciable fraction of the proflavine-induced mutant strains do in fact fail to produce plaques on ordinary media, as expected. Other proflavine-induced mutant strains, however, unexpectedly produced plaques even on unsupplemented plates.

The relative sites of these mutations have been established by genetic crosses. In all those strains that can produce plaques on ordinary media, the mutation is located in the terminal third of the lysozyme gene, while in the strains that cannot produce plaques, the mutation is in the other two thirds of the gene (*18*).

These results are entirely compatible with Crick's hypothesis and in fact tend to confirm it. That mutation in the terminal third of the gene results in the production of some lysozyme activity, while mutation in the rest of the gene results in no activity, suggests that the integrity of two thirds of the lysozyme molecule is essential for activity and that the terminal third of the molecule is not essential for some degree of activity. The level of lysozyme activity in those proflavine-induced mutant strains that form plaques is 1 percent or less (depending on the particular mutant strain) than the level of activity in the standard type strain.

Spontaneous revertants of several proflavine-induced, lysozyme-mutant strains have been isolated. As in the r_{II} mutations studied by Crick *et al.*, the reversions in several cases could be shown to be due to suppressor mutations located near the original mutation. Strains carrying the suppressor mutation alone lack lysozyme activity or else have greatly decreased amounts of lysozyme activity, as would be expected. The double mutant strains that arose as revertants could be shown in some cases to have levels of lysozyme activity similar to those of the wild-type strain, again as could be expected on the basis of Crick's hypothesis (*19*).

In some cases, genetic crosses between two independently isolated proflavine-induced lysozyme mutants produced wild-type recombinants and also pseudowild-type recombinants which could be shown to be the double mutant. The level of enzyme activity of the double mutant strains was again similar to the level of activity of the wild-type strain (*19*).

The most direct test of Crick's hypothesis is the comparison of the lysozyme of a wild-type and a pseudowild-type strain. A pseudowild-type strain carries two proflavine-induced mutations. Normally, for two mutations not of the acridine-induced type, two amino acids would be expected to be changed in the product protein. With acridine-induced double mutants that result in a pseudowild-type phenotype, all amino acids (and hence often more than two) between the sites of mutation would be expected to be changed.

The lysozyme produced in infected bacteria under the influence of the genome of phage T4 can be readily isolated. Very recently, Eric Terzaghi and Yoshimi Okada, working in my laboratory in collaboration with Akira Tsugita, of Osaka University, have examined the lysozyme produced by a normal strain and a double mutant strain. They have found that the lysozyme of the two strains differs with respect to a sequence of five amino acids.

These results (details of these experiments will be described in full elsewhere) confirm Crick's hypothesis and prove that the genetic code is a triplet code that is read sequentially from a given point at the beginning of the gene on to the end of the gene.

GENES AND CHROMOSOMES

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DISCUSSION

Reissig: I wonder how the polarity of the lysozyme gene compares with the polarity of other genes in this phage?

Streisinger: The question that Dr. Reissig asked concerns the direction of the translation in different parts of the phage chromosome, I believe. The direction of reading is probably the same in the case of the lysozyme and the head genes but these two are probably translated in a different direction from the r_{II} gene. The r_{II} gene is one that functions early, the head and the lysozyme protein appear late; it would be of interest to know whether the direction of translation is in general correlated with time of function in this system.

Allende: In the case of hemoglobin the work of Dintzis and Schweet has shown that the protein is synthesized starting from the aminoterminal and proceeding to the carboxyterminal end of the protein molecule. Since you find proflavine-induced mutants that form functional lysozyme and that are grouped at one end of the cistron, could you conclude that the carboxyterminal end of the molecule is not essential for activity in this enzyme?

Streisinger: There is a considerable latitude with respect to the substitution of amino acids at this end of the lysozyme molecule, but I do not think that this necessarily means that the end can be dispensed with.

Perry: Can we conclude that the existence of a class of mutants containing alterations near one end of the cistron but still producing functional lysozyme points to the fact that the lysozyme message is not part of a polycistronic message? If it were, then the information in subsequent messages would be garbled.

Streisinger: In the case of phage, no polycistronic groups that are transcribed into a single message are known as yet. In any event, it is not clear that a single base deletion or addition mutant that would shift the "reading frame" would interfere with the correct translation of the next cistron. We do not as yet know (at least I do not know) whether the "code" for beginning translation of a particular cistron has to be "in phase" with the translation of the previous cistron. In the case of some nonsense mutants that result in the interruption of the translation of one cistron, the correct translation of the next cistron is not interfered with.

Perry: Are these nonsense mutants of the proflavine type?

Streisinger: The nonsense mutants that are suppressible are not of the proflavine type; they do not involve additions or deletions of bases but rather changes from one base pair to another. The nonsense mutants are revertible by base analogues whereas proflavine mutants, of course, are not.

Basileo: Do you have any idea about the mechanism of addition or deletion of bases by proflavine?

Streisinger: No, I do not. The work of Lerman suggests that proflavine gets inserted into the DNA molecule between base pairs, thus stretching the molecule. Some recent evidence suggests that replication is not essential for proflavine mutagenesis, but that it occurs through a process involving recombination.

R. F. Grell: How many triplets are available for nonsense punctuation since the code is quite degenerate?

Streisinger: I do not think that one can answer that question at this time. It is clear that at least two different triplets exist for nonsense of the type that involves the interruption of translation and quite possibly more exist.

Reissig: How can you conceive of a universal code, identical or almost so throughout nature, but allow for divergent coding between closely related bacterial strains?

Streisinger: There are probably strong selective pressures that prevent changes in the code. The change in the case of the bacterial suppressor mutation may involve an s-RNA that has not previously been used to code for any particular amino acid.

Genetic Control of the *p*H 7.5 Esterase in Maize [1]

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FOR THE PAST 5 years our efforts have centered on investigation of the genetic control of an esterase in maize. A number of enzymes with esterase activity, specified by nonallelic genes, occur in maize plants. At least 10 different esterase bands can be identified by starch gel electrophoresis; the number varies in different tissues. We have concentrated our studies on 1 enzyme with an isoelectric point around *p*H 7.5. The gene controlling this enzyme has been designated E_1. The isoelectric point was established by determining the *p*H at which the enzyme specified by the most common allele, E_1^N, remained at the origin and did not migrate to either the anodal or cathodal pole. Unfortunately, up to now it has not been possible to correlate any phenotypic differences with the various alleles of the E_1 gene, and as a consequence the genetic analysis depends entirely on the techniques of electrophoresis. Seven alleles of the E_1 gene have been found. The mutant forms specify enzymes with altered charge which migrate at different rates.

1-Naphthylacetate was used as the substrate in these studies. The techniques for electrophoretic separation and the development of the zymograms have been described (*1, 2*).

Sucrose gradient centrifugation of partially purified preparations of the *p*H 7.5 esterase, combined with a reference enzyme of known molecular weight, has enabled us to set the molecular weight of the enzyme at approximately 95,000.

HYBRID ENZYMES IN HETEROZYGOTES

The enzyme exists as a dimer of which two types occur: autodimers composed of 2 identical monomers specified by the same allele, and allodimers composed of nonidentical monomers specified by 2 different alleles of the E_1 gene. The allodimers occur only in heterozygotes and are always associated with 2 autodimers. Thus, in homozygous plants only a single *p*H 7.5 esterase band is observed, whereas in heterozygotes 3 bands are seen. The allodimer or hybrid enzyme has a migration rate intermediate between those of the autodimers formed by the two alleles in the heterozygote (*2*).

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The pattern of association of the monomers in the *p*H 7.5 esterase system differs from hemoglobin and lactic dehydrogenase. In hemoglobin, the two alpha or beta polypeptides associated in the same tetramer are always identical, being specified by the same allele. Individuals suffering from sickle cell trait are heterozygotes carrying an Hb^A and an Hb^S allele. Only two types of hemoglobin molecules are formed; either with two B^A or two B^S polypeptides (*3*). Lactic dehydrogenase is also a tetramer, but polymerization can involve either identical monomers specified by the same gene or a mixture of monomers specified by two nonallelic genes (*4*). The *p*H 7.5 esterase lies between these two extremes; dimerization involves monomers specified by only one locus but not necessarily the same allele.

Monomers specified by the E_1 gene do not dimerize with other monomers formed by nonallelic genes. Only a single *p*H 7.5 esterase band is seen in E_1 homozygotes, and the positions of all other bands with esterase activity are not changed in strains carrying the various E_1 alleles. One allele of the nonallelic E_3 gene, E_3^F, produces an esterase with a migration rate identical to that formed by E_1^S (SS). The E_3 esterase is also a dimer and both the autodimer and allodimer enzymes are formed in E_3^F/E_3^S heterozygotes (*5*). However, dimerization does not occur even between E_1 and E_3 monomers which are similar in activity and charge.

Most of the heterozygous combinations involving the 7 E_1 alleles have been synthesized. In all the heterozygotes 3 *p*H 7.5 esterase bands, comprised of 2 autodimers and the allodimer, occur. In every case, the association between the monomers appears to be random. Preferential dimerization of identical monomers specified by the same allele would result in a relatively high concentration of the 2 autodimers as compared to the allodimer, but preferential dimerization of monomers specified by 2 different alleles would result in a relatively low concentration of the 2 autodimers. Neither condition has ever been encountered in our studies. The relative intensities of the 3 *p*H 7.5 esterase bands in heterozygotes always follow the distribution expected from random association of monomers, whether the analysis is of extracts from diploid tissue in which each heterozygous allele is represented only once or in triploid endosperm tissue in which 1 allele is present in 2 doses. In the latter, the relative intensities of the 3 *p*H 7.5 esterase bands depend on the direction of the cross, since the female parent contributes 2 sets of genes. Tetraploid seedlings carrying 3 different alleles E_1^F, E_1^N, and E_1^S, show 5 bands. This finding conforms with the dimer hypothesis. Six types of dimers are expected: FF, FN, NN, FS, NS, and SS; FS and NN have identical migration rates and appear as a single band.

In heterozygotes the two autodimer bands, depending on the allele combination, are not always of equal intensity. This may reflect a difference in gene activity so that less enzyme is being made by 1 allele, or a difference in the specific activity of the isozymes. The E_1 gene is functional in almost all actively growing tissues of the maize plant. The pH 7.5 esterase is formed in the leaf, root, embryo, endosperm, silk, cob, etc. In particular heterozygous combinations, *e.g.*, E_1^F and E_1^{N469}, the concentration of the NN dimer may be very low compared to the FF dimer in one tissue, while in another tissue the NN band is more intense than the FF band. Thus, either the 2 isozymes have different specific activities in various tissues of the same plant or the alleles have different relative activities. The latter is the favored hypothesis. Even in those heterozygotes containing unequal concentrations of the 2 autodimers, the intensity of the allodimer or hybrid enzyme band is consistent with what would be expected from random association of monomers.

DIFFERENTIAL INACTIVATION OF ALLELIC ISOZYMES BY UREA

As was mentioned previously, the hybrid enzyme formed in E_1^F/E_1^S heterozygotes has a migration rate identical with the enzyme formed in E_1^N/E_1^N homozygotes. According to the dimer hypothesis, these 2 isozymes are composed of different monomer combinations. The isozyme formed in E_1^N/E_1^N homozygotes is composed of 2 N monomers, whereas the hybrid enzyme of E_1^F/E_1^S heterozygotes is composed of 1 F and 1 S monomer. These 2 dimers, though identical in net charge, show a difference in inactivation by urea. The enzymes specified by the various alleles may differ in urea sensitivity as well as electrophoretic mobility. This difference has been observed only for enzymes synthesized in endosperm tissue, not in the sporophyte. The FF and SS isozymes formed in $E_1^F/E_1^F/E_1^F$ and $E_1^S/E_1^S/E_1^S$ endosperms, respectively, are quite resistant and are only slightly inactivated by 15-minute treatment with 6 M urea. However, the NN enzyme formed in $E^N/E^N/E^N$ endosperm is almost completely inactivated by the same treatment. The endosperm FS hybrid enzyme composed of 2 "resistant" monomers is only slightly inactivated by the urea treatment. Treatment of extracts of heterozygous endosperm containing both a sensitive and a resistant allele, such as $E_1^N/E_1^N/E_1^F$, show almost complete inactivation of the NN isozyme, only slight inactivation of FF, and intermediate inactivation of the FN allodimer.

The NN isozyme specified by the E_1^N allele functioning in the endosperm is sensitive to urea while the NN isozyme specified by the same allele in the plumule of the seedling is urea-resistant (*6*). This reflects a difference in the structure of the enzyme molecules themselves in the two tissues, as we have been able to rule out any influence of other components in the two extracts on the urea inactivation. The differential sensitivity is still maintained in mixtures of extracts from the plumule and endosperm. The FF isozymes of both endosperm and plumule origin are resistant to urea. Reciprocal mixtures were made of endosperm and plumule extracts containing the NN and FF isozymes. In both mixtures the FF enzyme was resistant to urea. However, the sensitivity of the NN enzyme was strikingly different in the reciprocal mixtures depending on whether it was of endosperm or plumule origin. The differential sensitivity of NN from the two tissues persists even after a considerable degree of purification.

The NN isozymes from the two plant parts probably do not differ in primary structure. They have the same net charge since their electrophoretic migration rates are identical. The difference is most likely in their tertiary structure or folding. The "thermodynamic" hypothesis of tertiary structure of proteins states that no *genetic* information other than that which specifies the primary structure (amino acid sequence) is required for the tertiary structure of the protein (*7*). A polypeptide of given amino acid sequence will assume the folded configuration which is thermodynamically most stable. However, the thermodynamically most stable configurations of the same polypeptides synthesized in diverse cellular environments may be quite different. Possibly, relatively minor differences in tertiary structure may be sufficient to account for a difference in urea sensitivity, and thus the result just presented would not necessarily be contrary to the widely accepted hypothesis for the genetic control of protein specificity.

IN VITRO INTERCONVERSION OF ALLELIC ISOZYMES

Recent work in our laboratory on the effect of sodium borohydride ($NaBH_4$) provides an explanation for the identity in the charges of the NN and FS isozymes. When extracts from plants homozygous for the E^F, E^N, or E^S alleles are treated *in vitro* with $NaBH_4$, the single *p*H 7.5 esterase isozyme is converted into a number of isozymes which in electrophoresis show up as a series of sharp, discrete bands all more negatively charged than the original isozyme (*8*). The number of new bands formed depends on the concentration of $NaBH_4$ used and the isozyme treated. Treatment of the FF isozyme results in a maximum of 6 bands, treatment of NN results in 4 bands, and treatment of SS results in only 2 bands. The conversion is always unidirectional to less positively charged isozymes. By the use of the overlapping technique in starch gel electrophoresis, we have been able to show that the new bands formed as a result of treatment with $NaBH_4$ have migration rates identical to the isozymes formed by the E_1^F, E_1^N, and E_1^S alleles, both the autodimers and the allodimers. Treatment of an FF extract results in bands corresponding in position to the FF, FN, NN (=FS), NS, and SS bands as well as a new, slowly migrating band that occupies a position between SS and the origin. After treatment of NN with $NaBH_4$, one finds bands at the positions of NN, NS, SS, and the slowest band. Treatment of SS results in only the original SS band and the very slow band.

The hypothesis advanced to explain these results is as follows: We postulate that the net charge of the amino acids making up the polypeptides is identical for the F, N, and S monomers. The 3 polypeptides may differ in amino acid composition, but the differences involve the replacement of one amino acid by another of like charge so that the net charge remains unchanged. We propose that the monomers differ in the number of positively charged side groups conjugated to the polypeptide, and that this accounts for the difference in migration rates. To conform with the data, we assign 3 such groups to the F monomer, 2 to N, and only 1 to S. Thus the FF dimers will have 6 positively charged groups, NN 4, and SS 2. Of the allodimers, FN would have 5, FS 4, and NS 3 such groups. NN and FS would each have 4 positively charged groups conjugated to the polypeptides (2 + 2) and (3 + 1), respectively, and hence show equal migration rates. If the action of $NaBH_4$ were simply to remove or neutralize varying numbers of these positively charged groups, unidirectional isozyme interconversion would result.

The $NaHB_4$ mediated band shift or isozyme interconversion requires the presence of aldose sugars. If crude enzyme extracts are dialyzed for 48 hours, no band shift results from the $NaHB_4$ treatment. The shift is, however, observed when aldohexoses or aldopentoses are added to the dialyzed extract before $NaBH_4$ treatment. The keto sugars sorbose and fructose are not effective, neither are the polyols sorbitol, dulcitol, mannitol, and arabitol, nor other sugar derivatives such as glucosamine and galactosamine. These compounds not only are ineffective in producing the shift with $NaBH_4$ but also are competitive inhibitors and prevent the shift from occurring when added together with the aldose sugars. Some compounds, such as sucrose, methyl glucoside, and glucose-6-phosphate neither cause nor protect against the shift.

These studies are still under active investigation. We believe that the results obtained to date indicate a specific association of the sugars and sugar derivatives with the *p*H 7.5 esterases. Other esterases in the extracts are not affected in like manner. Our working hypothesis is that the $NaHB_4$ causes a transfer of the positively charged group from the enzyme to the sugar. Some of the ineffective compounds tested, such as the polyols and keto sugars, are believed to have the specificity for association but are not active as receptors for the positively charged groups. Hence, they do not cause the shift, and moreover, prevent its occurrence when present together with the aldohexoses, since they compete for the association sites on the enzyme molecules. The other class of related compounds that neither cause nor protect against the shift may lack the steric configuration for association with the sites on the enzyme.

Seven alleles of the E_1 gene have been found but treatment of the FF, NN, or SS with $NaBH_4$ does not result in conversion to isozymes formed by the other alleles even though some of them specify isozymes with migration rates intermediate between FF and SS (*8*). The E_1^L, E_1^R, E_1^T, and E_1^W alleles form a second series. The LT allodimer has a migration rate identical to the RR autodimer; RW is like TT. Treatment with $NaBH_4$

results in isozyme interconversion within this series. The charge difference between L, R, T, and W is the same as that between F, N, and S. This is shown by the fact that the migration rate of the FW allodimer is identical to that of the NT allodimer. These results can best be accounted for by postulation that the isozymes in the two series differ in the net charge of the amino acids making up the polypeptides. The 7 autodimers thus differ in amino acid composition and/or the number of positively charged side groups conjugated to the polypeptides.

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Gene Differentiation [1, 2]

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SUMMARY

Differentiation between homologous genes of *Salmonella typhimurium*, *Salmonella montevideo*, and *Escherichia coli* was studied by determining recombination frequencies of genetic markers observed in crosses (transductions) between these species.—Nat Cancer Inst Monogr 18: 15–20, 1965.

IN MY REPORT at this Symposium I reviewed evidence accumulated in our laboratory during the past decade as it may relate to the evolutionary events responsible for observed similarities and divergencies between the genetic material of *Salmonella typhimurium* and that of *Escherichia coli*, *Salmonella montevideo*, and *Salmonella abony*. Some of the data, particularly those relating to similarities of gene arrangement in *S. typhimirium* and *E. coli*, have recently been discussed at a Symposium on "Evolving Genes and Proteins" held at the Institute of Microbiology, Rutgers University, New Brunswick, New Jersey, and are being published in the proceedings of that Symposium (*1*). The present paper summarizes material already presented in detail and describes more recent studies of genetic divergence between *S. typhimurium* and *S. montevideo* or *S. abony*.

Similarity and divergence of genetic material—a summary.—Comparison of the genetic maps of *S. typhimurium* and *E. coli* reveals a strikingly high degree of homology between the two genera in gross structure, that is, in the locations and functions of their genes. No well-substantiated case of chromosomal rearrangement has been discovered, although more than 100 genes have been mapped in each organism. Thus, it appears that in the evolutionary differentiation between these two genera rearrangements have occurred much less frequently than in higher organisms such as *Drosophila*. The phenomenon may be explained by the finding that, in both *S. typhimurium* and *E. coli*, genes controlling related functions are often clustered together. Seventeen such clusters, distributed along the genome, have

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[2] Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.

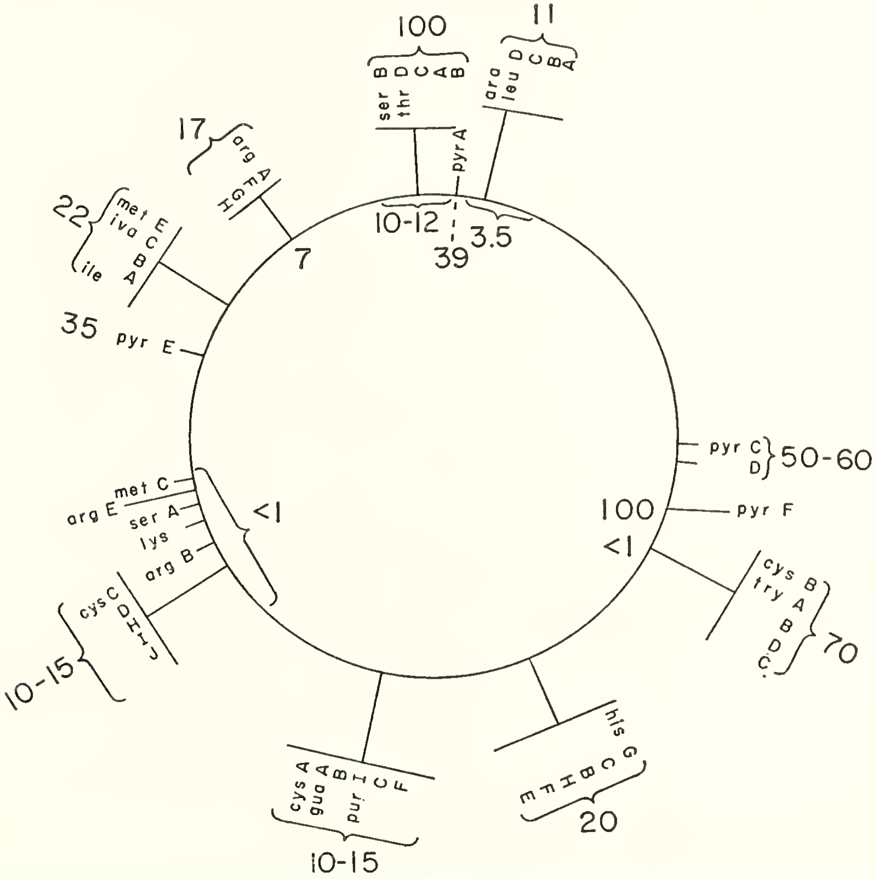
[3] I gratefully acknowledge the help of Miss Agnes C. Fisher in editing the manuscript.

been found in *S. typhimurium.* We can assume that the gene arrangement represented by a cluster, in order to persist, must offer some selective advantage to the organism, so that chromosomal rearrangements, which are likely to break up clusters, will be selected against. Therefore the high degree of homology of gene arrangement observed in the two species can be explained by assuming that a common progenitor also had clusters, which were responsible for retention of its gene arrangement in species and subsequently genera—derived from it.

A technique has been developed for determining the frequency of incorporation of an *E. coli* genetic marker into the homologous region of the *S. typhimurium* chromosome. First, hybrids are obtained by conjugation between an *S. typhimurium* recipient and an *E. coli* Hfr. Then, in transduction experiments utilizing phage P22, chromosomal fragments of *E. coli* origin are transferred from hybrid donor cells into suitably marked *S. typhimurium* recipient cells, where they are sometimes incorporated into the chromosome. In general, the frequency of incorporation is considerably lower than that of *S. typhimurium* chromosomal fragments (control experiments). This finding has been interpreted as an indication of structural divergence between the chromosomes of the two genera. In view of the unexpectedly high degree of correspondence in gene order, just noted, it is inferred that this divergence is due to differences at the molecular level rather than to differences in gross chromosomal structure, and that genes may be homologous as to function and yet nonhomologous in molecular structure. Thus, if homology of molecular structure is essential for integration, two genes although responsible for synthesis of the same enzyme may fail to recombine freely.

Extent of molecular homology between genes of S. typhimurium and E. coli and S. montevideo.—Estimates of the degree of molecular homology between *S. typhimurium* genes and corresponding genes of *E. coli* (or *S. montevideo*) are obtained by measuring the frequency of incorporation of genetic markers of *E. coli* (or *S. montevideo*) into *S. typhimurium.* Measurement is accomplished by determining the frequencies of transductants in crosses between *S. typhimurium* recipients and *E. coli* (or *S. montevideo*) donors, and comparing them with the transduction frequencies observed when both recipient and donor are *S. typhimurium.*

Tests involving genes located in different regions of the chromosome show that the degree of homology is not uniform throughout. Text-figure 1 summarizes results of such tests. Numbers inside the circle indicate degrees of homology between certain gene loci of *S. typhimurium* and *E. coli;* those outside the circle refer to loci of *S. typhimurium* and *S. montevideo.* It is evident that some genes—in *E. coli, pyrF;* in *S. montevideo, pyrA* and genes of the *thr* transducing fragment—show a high degree of homology in crosses with comparable genes of *S. typhimurium,* whereas others, particularly *E. coli* genes located in the *cysC* and *try* clusters, demonstrate very little homology. It appears also that genes located in the same transducing fragment show similar degrees of homology, as was predictable from the results of an earlier study of transduction frequencies for markers carried by composite *S. typhimurium-E. coli* transducing fragments (*2*). In those experiments, genes located in the Salmonella portion of a fragment were incorporated with about the same frequency as if the whole fragment were Salmonella, whereas the incorporation of genes located in the *E. coli* portion was about 40 times more frequent than the integration of genes of an analogous all-*E. coli* fragment. These results are explained in terms of the effect exerted by molecular homology on the synaptic forces that bring together corresponding regions of two chromosomes. They support the assumption that the effect of differences in molecular homology on transduction potential may be greatly modified when genes are located in the same transducing fragment.



TEXT-FIGURE 1.—Partial linkage map, showing the positions of gene loci that have been tested for homology. Numbers *inside* the circle indicate percentages of homology between *S. typhimurium* and *E. coli;* numbers *outside* the circle indicate degrees of homology between *S. typhimurium* and *S. montevideo.*

On the whole, the data of text-figure 1 indicate a higher degree of homology between *S. typhimurium* and *S. montevideo* than between *S. typhimurium* and *E. coli,* as should be expected if such homology is related to evolutionary differentiation.

GENES AND CHROMOSOMES

In tests of the *cysC* region, *S. abony* was included in addition to *S. montevideo* and *E. coli*. *S. typhimurium* has somatic antigens i, 4, 5, and 12 and flagellar antigens il-2; *S. abony* has somatic antigens i, 4, 5, and 12 and flagellar b-e, n, x; whereas *S. montevideo* has no antigens in common with *S. typhimurium* (somatic 6, 7; flagellar g, m, s). Thus we may assume that *S. abony* is more closely related than *S. montevideo* to *S. typhimurium*. Genetic tests of *cysC, D, H, I, J* showed 10 to 15 percent molecular homology between *S. typhimurium* and *S. montevideo* (text-fig. 1), but about 80 percent between *S. typhimurium* and *S. abony*. Here again, homology values are in agreement with the taxonomic relationship.

RESUMEN

Se ha diseñado un método para probar la frecuencia de intercambio de material genético entre pequeños segmentos del cromosoma de *Salmonella typhimurium* y los cromosomas de otras especies de bacterias capaces de hibridarse con *S. typhimurium*. El procedimiento seguido envuelve primero la obtención de los híbridos entre *S. typhimurium* y otras especies y luego la trasducción de un fragmento del cromosoma del híbrido a células marcadas de *Escherichia coli*, por medio del fago P_{22}. En esta forma es posible comparar las frecuencias de incorporación de secciones dadas de cromosomas provenientes de la otra especie o de *S. typhimurium*.

En general, se halla que la frecuencia de incorporación del material genético de otra especie en *S. typhimurium* es menor que la incorporación de su propio material genético. Se discutirán evidencias que indican que esto es debido a divergencias en la estructura molecular del material genético más que a divergencias en su estructura gruesa.

Experimentos con otras tres especies han demostrado que el más alto grado de homología fué hallado entre *S. typhimurium* y *S. abony*, en menor grado entre *S. typhimurium* y el más bajo fué evidenciado entre *S. typhimurium* y *E. coli*.

Se ha observado también que diferentes regiones de los cromosomas difieren en su homología.

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DISCUSSION

Streisinger: You would expect that in abortive transduction, nonhomology would not have any effect. Is abortive transduction still normal in *Salmonella-Escherichia coli* transduction?

Demerec: Yes, abortive transduction is normal.

Pavan: Concerning the absence of inversions and transpositions in *Salmonella* chromosomes, you say that you do not find such rearrangements because there *is* an adaptation in the distribution in the system of clusters. I think a similar situation is also found in chromosomes of higher organisms like *Drosophila*. When new rearrangements occur, I think they are at a disadvantage and only adaptation could perpetuate them. As in this chromosome you probably have only one molecule and the message is going in one direction, could it be possible biochemically to change the direction or part of the message in such a way that one section would go in one way and the other in the opposite direction? Would you not think that one of the main reasons why inversions and transpositions would not be found is because there is a single molecule, while in higher organisms there are several different molecules in the chromosome?

Streisinger: I am certainly not a biochemist, but nevertheless I think that in phage, as I mentioned before, there is one chromosome. Dr. Edgar will discuss whether it is one molecule or not, but, at any rate, phage T4 has different genes, read in different directions. There are three genes: lysozyme, head protein, and r_{II}. Lysozyme and r_{II} are read in different directions, so presumably one is read from one strand and the other is read from another strand, of course at very different positions.

Demerec: P. Margolin, K. Sanderson, P. Hartmann, and others obtained good evidence showing that all clusters in *Salmonella typhimurium* are not oriented in the same direction, which indicates that the reading of the message is not continuous.

I believe that an inversion can occur in *Salmonella*. Jacob and Wollmann report in their book two inversions induced by a mutagen, and I hope that before long we shall find inversions in our strains of *S. typhimurium* and *E. coli*. However there seems very little doubt that, in our material, chromosomal rearrangements are much less frequent than might be expected from work with higher organisms, and I think frequent clustering of genes is greatly responsible for this condition.

Reissig: Is it known whether the degree of clustering is as high in *E. coli* as in *Salmonella?*

Demerec: I think it is, but unfortunately in regard to clustering the genetic analysis of *E. coli* is so much behind the *Salmonella* analysis, we cannot be certain about it.

Schultz: I am somewhat troubled by the evolutionary explanation of the lack of inversions in your bacterial stains. If there were a selective advantage in the clusters, there could still be plenty of opportunity for inversions *between* clusters, maintaining them in their original state. What your interpretation therefore implies is that the constellation of different clusters in the chromosome is important, but, since you also say that the message can be read in different directions in different clusters, that part of the interpretation is complicated. I believe that we are really dealing with somewhat different systems, in which the arrangements have various degrees of complexity. In these "DNA chromosomes" of bacteria, the possibility of rearrangement is restricted as compared with the DNA protein complexes with which we have to deal in organisms with the mitotic type of cell division, where the mechanism permits more latitude in the arrangements of the different units. This may be the kind of thing Dr. Pavan was getting at. I myself would not want to enter into a discussion of how many molecules there are in the chromosome, since it involves both the definition of a molecule and its implications for the definition of the replicating units. But I still cannot be quite content with the evolutionary explanation of the absence of inversions.

Demerec: We have analyzed only 82 genes, a small fraction of the total. What further analysis would show is hard to predict. I would not be surprised if there was some arrangement of super clusters which keep the clusters together. I doubt that lower complexity of bacterial chromosomes is responsible for the lack of divergence in gene order. If that were so, then a situation found in *Salmonella* should be general for all bacteria. However, fragmentary experimental evidence indicates that, in certain bacteria, clustering, if it occurs at all, is less frequent than in *Salmonella*.

Lindsley: I wonder if it would be possible to reconcile this dilemma by simply postulating that the entire circular chromosome is DNA and that it is practically impossible to insert two breaks to cause a rearrangement without interrupting a portion of the code in such a way as to produce a mutation. In such a system it is easy to understand why selective pressure has not allowed rearrangements, whereas in higher organisms, where there may be intercodonic material, breaks that do not interrupt the code are possible.

Demerec: If it is correct that, experimentally, inversions can be produced by ultraviolet rays, it would mean that there is nothing against their being formed. It is just that they cannot persist in nature.

Wolff: I would like to ask Dr. Streisinger a question in regard to his answer to Dr. Pavan. I wonder if, because the chromosome is read in two directions, it necessarily means that both strands are read? I think that in phage alpha Graziozzi finds that unless he treats with ultraviolet only one strand is read. Are you presupposing that there is only one enzyme for the transcription?

Streisinger: I should have thought the easiest way to imagine this is that the different strands are read in different regions. One can also assume that the same strand is read in different directions. It is somewhat harder for me to picture.

Pavan: You said that you could have a cluster that goes in one direction and another that goes in the opposite one. If you compare *Salmonella* with *E. coli*, can you find a corresponding cluster going in one direction in one species and in the other direction in the other species?

Demerec: Published data are not available to make such comparisons.

Neurospora Mutants Deficient in Aspartic Transcarbamylase [1, 2]

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SUMMARY

This report deals with mutants selected as suppressors of *arg2* and with the metabolic interactions underlying this suppressive effect. These mutants are deficient in aspartic transcarbamylase, and all appear to affect the corresponding structural gene, in spite of a marked discontinuity in the spectrum of their phenotypes. Enzyme activity can be regenerated by complementation *in vivo* or *in vitro*. Treatment with 2-mercaptoethanol is required in the latter case. Complementation yields a very labile enzyme compared with the wild type, and this lability is greater for the product of complementation *in vitro* than for the product *in vivo*. The significance of these findings for the mechanism of interallelic complementation is discussed.—Nat Cancer Inst Monogr 18: 21–31, 1965.

OUR INTEREST in mutations affecting the enzyme aspartic transcarbamylase (ATC) of *Neurospora* was awakened by the discovery of a simple screening technique (*1*). This technique proved invaluable for the isolation of large numbers of *pyr3N* mutants, *i.e.*, mutants at the structural gene for ATC. Furthermore, it was remarkably sensitive, for it permitted selection of mutants with only slight impairment of ATC activity (subthreshold or *pyr3*^{su} mutants).

In this paper we shall first discuss the aforementioned technique and its metabolic background. Studies on the mutants will then follow: Subthreshold mutants will be discussed first and the more extreme types later. In this latter context, particular attention will be devoted to the regeneration of ATC activity by complementation *in vivo* and *in vitro*.

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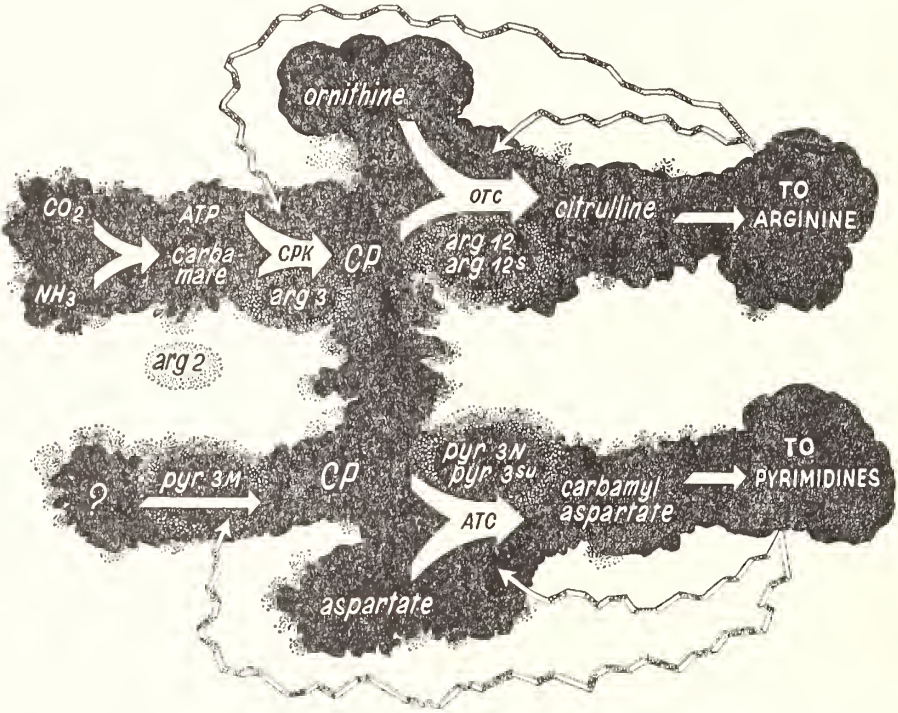
BIOCHEMICAL BASIS OF SCREENING PROCEDURE

Carbamyl phosphate (CP) is an intermediate for the synthesis of both arginine and pyrimidines in *Neurospora*, but a different CP pool is utilized for each of these two pathways (*2*, *3*). This is illustrated in text-figure 1, with the additional feature that the two pools appear united by a blotch. The artist, Mr. Pérsico, had good reasons for drawing the text-figure in this manner, as shall be clear directly.



TEXT-FIGURE 1.—Biosynthesis of arginine and pryimidines in *Neurospora*. This scheme summarizes the work of various laboratories (*2–11*). Enzymes are indicated *inside* the *arrows*, and controlling genes are *encircled with dots*. The location of *arg2* presents a puzzle: *In vivo* it blocks the synthesis of CP along the arginine path, but *in vitro* it displays carbamyl phosphokinase activity. The biochemistry of the step controlled by *pyr3M* is unknown. The *wavy arrows* indicate repression effects. *Abbreviations:* CP, carbamyl phosphate; ATP, adenosine triphosphate; ATC, aspartic transcarbamylase; OTC, ornithine transcarbamylase; CPK, carbamyl phosphokinase.

We can infer that the two CP pools are distinct because *arg2* and *arg3* mutants are arginine-dependent but pyrimidine-independent, whereas *pyr3M* mutants require pyrimidines but no arginine. [Further evidence for the separation of the pools has been presented by Davis (*2*)]. The same type of evidence can be used to show that under certain conditions the two CP pools can undergo exchange: The arginine requirements of *arg2* or *arg3* are suppressed in the presence of mutations reducing ATC activity (*pyr3N* or *pyr3*^{su}) (*7, 10*), and the pryimidine requirement of *pyr3M* is suppressed by mutations reducing ornithine transcarbamylase (OTC) activity (*arg12*^{s}) (*6, 8*).

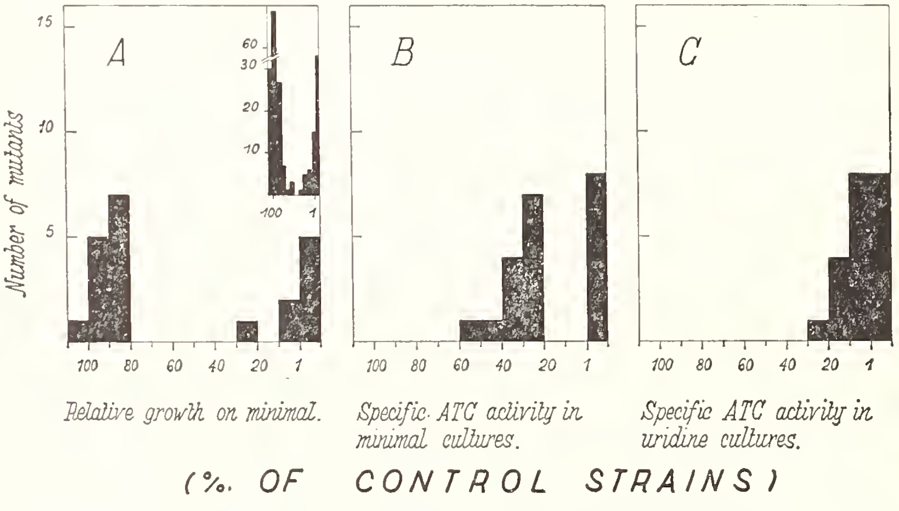
This simple explanation of the suppressive effects of a decrease in ATC or OTC is supported by additional evidence (*3, 4, 7, 11*) and is logically consistent. Indeed, if the rate of the reaction utilizing CP along a pathway is reduced by mutation relative to the rate of the synthetic reaction, accumulation of CP is expected. Flow of CP into the pool belonging to the other pathway would be a likely sequel to this accumulation, provided pool segregation is not strict but results from preferential channeling along each path. Such channeling could occur, for instance, if the enzyme activities for the synthesis and utilization of CP along at least one of the pathways are localized in a particular cell structure or molecule (*7*). It is also possible to have channeling even if all enzyme activities are homogeneously distributed, but the two paths never function simultaneously. While this is only a theoretical possiblility, it is interesting that cyclic regulation of enzyme synthesis and activity has been reported in some systems (*12, 13*).

It is easily seen how the suppression of *arg2* by mutants affecting ATC can be the basis for the screening of ATC mutants. In practice, microconidia from an *arg2* strain are treated with the desired mutagen and plated on media supplemented with uridine, but not with arginine. Two kinds of mutants grow on such plates: backmutants at *arg2*, which are very rare, and forward mutants with impaired ATC activity (*10*). In the original procedure the residual growth ("leakage") of *arg2* in the absence of arginine was inhibited by the addition of lysine and canavanine to the screening medium. In a more recent modification of the method, the plates were placed in desiccators containing potassium hydroxide solution (Reissig, unpublished data), which thus lowered the carbon dioxide tension to the point where *arg2* strains will not leak, but suppressed strains can still grow (*11*).

SUBTHRESHOLD MUTANTS

According to the previous discussion, reduction in ATC, followed by accumulation and overflow of CP, leads to suppression of *arg2*. To what extent must ATC be reduced before this mechanism is put into effect and a mutant becomes detectable by our screening procedure? As will be seen, a 40 percent reduction, *i.e.*, 60 percent of normal activity, will suffice for detection, a figure underscoring the sensitivity of the method. This sensitivity is particularly remarkable because the ATC activity has to decrease to 20 to 30 percent before the pyrimidine requirement for growth starts to appear. The latter figure (20–30%) is close enough to the figure of 40 percent assigned by Donachie (*14*) to the level at which ATC becomes limiting for growth in the absence of pyrimidines in *pyr3N*/*pyr3N*$^{+}$ heterokaryons, considering that the strains used by Donachie and ourselves differed in genetic background. Thus, taking 25 percent as the approximate point where auxotrophy begins in our strains, we can see that in the range between 60 and 25 percent ATC we are selecting typical "subthreshold mutants" (*15*), *i.e.*, mutants with a genetic block too lax for auxotrophy. The lack of knowledge about subthreshold mutants in general has fostered our interest in this particular sample.

The ATC subthreshold mutants are called $pyr3^{su-arg2}$ ($pyr3^{su}$) since they are detected as suppressors and are alleles of the more extreme ATC-deficient auxotrophs (*16*). At one time (*10*) it was suspected that subthreshold mutants ($pyr3^{su}$) and auxotrophs (*pyr3N*) might be basically different despite their analogous biochemical and mapping characteristics, because we obtained many extreme auxotrophs—with hardly any residual growth on minimal medium—and full prototrophs, but very few mutants of intermediate phenotypes. This discrete distribution is illustrated in text-figure 2A, where the inset refers to 196 different mutants and the main figure to a representative sample thereof. This same sample was used for the determination of ATC activities, with the results shown in text-figure 2B. The bimodal distribution of phenotypes is again in evidence: $pyr3^{su}$ mutants have 20 to 60 percent of the normal ATC specific activity, and *pyr3N* mutants have practically none, with no mutants in between.



Text-figure 2.—Frequency distribution of different phenotypes among aspartic transcarbamylase (ATC) mutants. All values are expressed in percent, and backmutants at *arg2* (average) are taken as control (100%). Strains tested are $arg2pyr3^{su}$ or *arg2pyr3N* originated by treatment of $arg2pyr3^{+}$ with nitrous acid or ethyl methane sulfonate. Phenotypes plotted: *A*—Growth in the absence of pyrimidines relative to growth in its presence (growth rate in minimal medium + growth rate in minimal medium + arginine/growth rate in minimal medium + uridine + growth rate in minimal medium + arginine and uridine); methods as in (*10*), except that linear growth rate was used in calculations. The inset refers to 196 mutants and the body of the figure to a sample of 21 mutants used also for *B* and *C*. *B*—Specific activity of ATC in cultures from liquid minimal medium. Methods are described elsewhere [(*17*); Jobbágy, unpublished data]. *C*—Same as *B*, but medium supplemented with enough uridine for full repression (1 mM/liter).

Yet the proposal that *pyr3*^{su} and *pyr3N* might differ in quality, *e.g.*, that one be the structural gene and the other the regulatory, turned out to be quite rash, because the obvious consequences of repression of ATC by pyrimidines (*cf* text-fig. 1) were overlooked. Clearly, reduction of the turnover number of ATC by mutation will lead to a lower pyrimidine pool and thus to stimulation of ATC synthesis. This will tend to restore normal activity within the capabilities of the regulatory system, which functions as a buffer both for end-product concentration and ATC specific activity. According to this view, *pyr3*^{su} mutants would be "buffered upwards" by derepression, and thus should experimentally show repression by uridine to a greater extent than wild strains. This is exactly what is found [5] (Jobbágy, unpublished data). Consequently, if we measure ATC activities under excess uridine, this preferential repression of *pyr3*^{su} mutants causes the bimodal frequency distribution to disappear (text-fig. 2C).

A general conclusion is that "leaky" mutants of any locus subject to end-product repression are expected to be an elusive class of mutants: They may be easily pushed into the subthreshold class by derepression.

AUXOTROPHS

Suppression is one of several ways by which *pyr3N* mutants can be grown without supplying pyrimidines to the medium. Thus, reversion to pyrimidine independence has yielded full revertants—analyzed genetically as backmutants or mutations close to *pyr3N*—and partial revertants due to mutation at an unlinked suppressor locus [(*1*); Reissig, unpublished data]. We have not investigated the biochemistry of this suppressor, but Woodward and Schwarz (*8*) studied a similar suppressor mutation that markedly reduced the normal level of OTC. Reference to text-figure 1 suggests how this reduction might facilitate the growth of a *pyr3N* "leaky" mutant under certain circumstances, although more work will be necessary before we can say with any certainty that this explanation is correct.

Inspired by the work of Champe and Benzer (*18*) on phage and Barnett and Brockman (*19*) on *Neurospora* Gampel and Reissig (*20*) tested base analogues to see if they would induce phenotypic reversion of *pyr3N* mutants. Ten of 12 "leaky" mutants gave slow but positive responses to 5-fluorouridine (from Hoffmann-La Roche Inc., Nutley, N.J.) or 8-azaguanine. But all 11 "nonleaky" mutants tested gave negative responses, even adding traces of uridine to allow for incipient growth. The contrasting behavior of "leaky" and "nonleaky" mutants suggests that an allelic protein is required for a positive response. These results were in accordance with theoretical expectations, but when the ATC activities of two 8-azaguanine-grown *pyr3N* cultures were analyzed by Augusto A. García (unpublished results), the unanticipated fact was that these cultures had only traces of ATC activity—the same level found in uridine cultures. On the other hand, García found that the specific activity of OTC was reduced to less than one half its usual value in the presence of 8-azaguanine. It may be that the phenotypic suppression effected by 8-azaguanine in these two mutants is of the same sort as the previously discussed genetic suppression studied by Woodward and Schwarz. This is just a preliminary explanation of our results, but it is useful as a reminder that suppression by base analogues may also be indirect, *i.e.*, an effect on the transcription—as current theory postulates (*18*)—of genes other than the one directly under consideration.

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[5] A further argument favoring the view that *pyr3*^{su} and *pyr3N* differ only in the extent of ATC damage is the observation that mutant KΦ493–50, of intermediate growth rate between *pyr3*^{su} and *pyr3N* strains, classified in the alpha complon and thus affecting the structural ATC gene (to be described), exhibits the high repressibility by uridine typical of *pyr3*^{su} strains.

Another method for growing the *pyr3N* mutants on minimal medium is complementation.

COMPLEMENTATION *IN VIVO* AND *IN VITRO*

Four complementation groups (complons) have been identified at the *pyr3N* locus, such that mutants in one complon will form pyrimidine-independent heterokaryons with mutants in another (*10*). The ATC activity was restored to levels ranging from 5 to 70 percent of the control strains in heterokaryons representing all 6 possible complon combinations. For further studies we selected a pair of mutants, K0492–68 and K0493–61, belonging to the alpha and beta complons, respectively. All results to be presented refer to this pair. We tried to obtain complementation *in vitro* *i.e.*, restoration of enzyme activity by mixing extracts from the individual mutants. The early attempts were only partially successful insofar as the mixed extracts had activities only slightly superior to the residual activities of the individual extracts. Various treatments expected to promote the reversible dissociation of polypeptide chains were then tried. Neither acidification nor urea treatment was helpful, but definite success was achieved with 2-mercaptoethanol (table 1).

The results of a number of similar experiments can be summarized in terms of specific ATC activity per mg protein as follows: Complementation *in vitro* yielded 3 to 4 units, complementation *in vivo* 6 to 8, and *pyr3N*$^+$ strains 20 to 30 units. Unfortunately, the true molecular efficiency of complementation cannot be calculated from these figures, since the extent of derepression in the different cultures is difficult to assess.

The finding of complementation *in vitro* and its facilitation by mercaptoethanol, an agent known to cause dissociation of disulfide bridges, is in good agreement with the prevalent idea that complementation is due to the interaction between protein subunits (*21*). Two models are usually considered for this interaction. The first model assumes an enzyme composed of dissimilar subunits, which may be called *A* and *B*. Com-

TABLE 1.—Regeneration of aspartic transcarbamylase (ATC) activity by complementation *in vitro*

Source of extracts*	ATC specific activity	
	Untreated	Treated with 2-mercaptoethanol
$pyr3N^{+}$	25	21
$pyr3N_{\alpha}$	0. 11	0. 15
$pyr3N_{\beta}$	0. 16	0. 20
$pyr3N_{\alpha}+pyr3N_{\beta}$	0. 25	3. 3

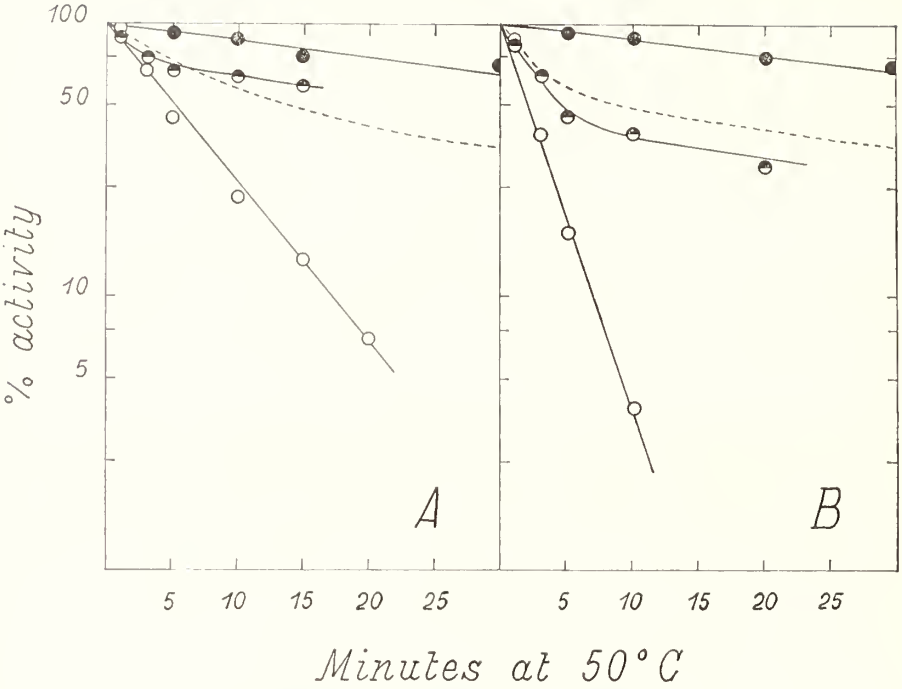
*Extracts were purified by ammonium sulfate precipitation, absorbed on calcium phosphate gel, eluted with phosphate buffer, and reprecipitated with ammonium sulfate (*17*). They were then mixed when needed, treated with 1 M 2-mercaptoethanol in 0.04 sodium acetate buffer, *p*H 5, for 30 minutes at 0 to 4° C, brought to *p*H 8.3 with 0.4 M Tris buffer-glycine, supplemented with 10^{-3} M Mg^{++}, and incubated 30 minutes at 0 to 4° C. Enzyme activities were then assayed under standard conditions (*17*) and are expressed in units (μmoles carbamyl aspartate formed per hour at 35° C) per mg protein.

plementation would then occur between A^- and B^- mutants because they contain intact B and A subunits, respectively. The second model ("intracistronic complementation") considers an enzyme composed of n identical monomer subunits (An), which by mutation become defective, *e.g.*, A' or A''. Complementation occurs because in the $A'_{n-m}+A''_{m}$ hybrid the two defective subunits will compensate for their shortcomings. The two models may be distinguished experimentally on the basis that the enzyme obtained by complementation should be identical to the wild-type enzyme in the first model, but different from it in the second.

As shown in text-figure 3, the enzymes obtained by complementation (lower curves), whether *in vivo* (text-fig. 3A) or *in vitro* (text-fig. 3B), differ markedly from the normal enzyme (upper curves) in their thermal stability. The behavior of the mixtures of labile and stable preparations (intermediate curves), which closely parallels the expected values for the sum, is proof that the differences reside in the enzymes themselves and not in impurities modifying their stability. These results clearly favor the intracistronic complementation model. They also show that *pyr3N* mutants involve the structural gene for ATC.

Our results with ATC are very similar to those of other authors who studied complementation in 6 different enzyme systems (*22–27*) and found in each case differences in heat stability between the normal enzyme and that obtained by complementation. The intracistronic model appears therefore to be a widespread mechanism for complementation. It is of interest that our results and those of the authors cited are also analogous in that complementation yields in every case an enzyme *more labile* than the normal enzyme. This might have been anticipated on evolutionary grounds: Enzyme stability must have an adaptative value in the wild type, but the stability of the heterokaryotic enzyme—a biological freak—is randomly determined by its physical chemistry.

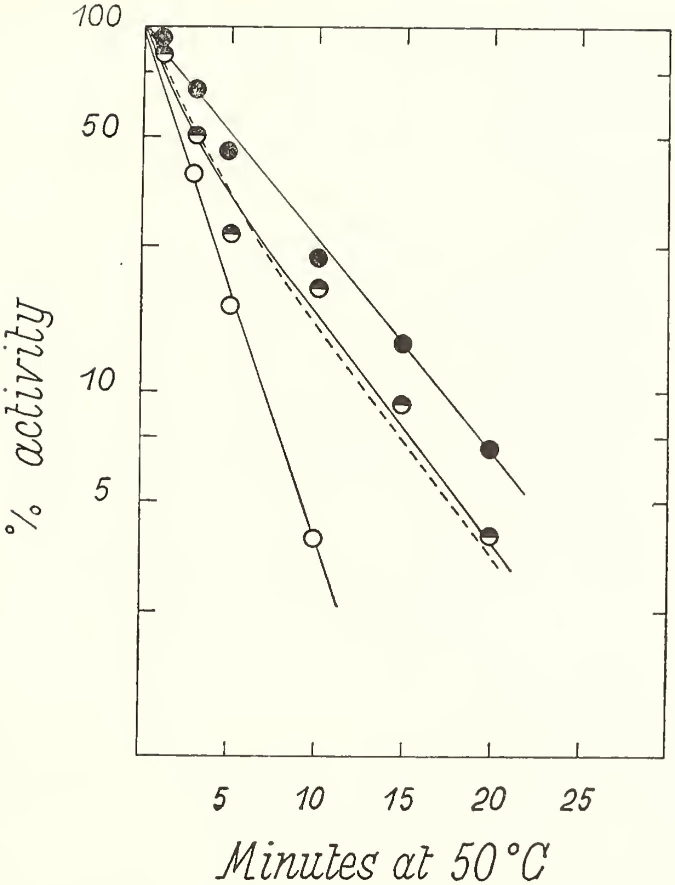
We wish to ask one more question about this enzyme freak with respect to the problem of how the protein subunits are assembled *in vivo:* Is the process *in vivo* the same as that effected *in vitro* subsequent to treatment



TEXT-FIGURE 3.—Heat stability of the aspartic transcarbamylase (ATC) obtained by commentation and of the wild-type enzyme. All enzyme preparations were pretreated with 2-mercaptoethanol, as in table 1, for the sake of uniformity. The behavior upon subsequent heat treatment was not affected by this procedure. These preparations, containing mercaptoethanol, buffer, and Mg^{++}, were heated at 50° C for various times, and ATC activity was subsequently assayed (*17*) and plotted as percent of the activity in the unheated aliquot. *Solid circles:* wild-type enzyme. *Open circles:* in *A*, enzyme from heterokaryon; in *B*, enzyme obtained by complementation *in vitro*. *Half-solid circles:* experimental points for the mixture of wild-type and heterokaryon enzyme in *A* or wild type and enzyme obtained by complementation *in vitro* in *B*. *Dashed lines:* calculated activity for former mixtures on assumption that no interaction occurs. Data from two different, typical experiments; some are also included in text-figure 4.

with 2-mercaptoethanol? This question can be answered by a comparison of the two products. As shown in text-figure 4, their heat stability is clearly different, and the behavior of the mixture (intermediate points) indicates that this difference is a property of the enzyme molecules themselves.

We have not yet been able to examine exhaustively the possibility that the difference in stability may be due to differences in the proportions of the two kinds of mutant subunits, but preliminary experiments lend no support to this alternative. Therefore, it appears that the mechanism for the interaction of protein subunits *in vivo* differs from the mechanism *in vitro* in the present system. This is not in itself surprising, although studies on complementation have often failed to provide evidence for this type of behavior (*24*). There are exceptions, however, as illustrated by



TEXT-FIGURE 4.—Comparison of the heat stability of the products of complementation *in vivo* and *in vitro*. *See* legend of text-figure 3 for all experimental details. All points are from the same experiment and include treatment with 2-mercaptoethanol. *Solid circles:* enzyme from heterokaryon. *Open circles:* enzyme obtained by complementation *in vitro*. *Half-solid circles:* experimental points for the mixture of the former two. *Dashed line:* calculated activity for same mixture with the assumption that no interaction occurs.

the work of Suyama and Bonner (*27*). Furthermore, Zipser and Perrin (*28*) found that complementation *in vitro* with *Escherichia coli* beta-galactosidase can be accelerated using a ribosomal-bound fraction. These interesting findings furnish us with favorable systems to explore the manner in which the protein subunits are assembled in the living organism.

RESUMEN

Se describen mutantes supresores de *arg2* y las interacciones metabólicas que constituyen la base de este efecto supresor. Estos mutantes son deficientes en la enzima aspártico transcarbamilasa, y todos parecen afectar al gen estructural correspondiente, pese a la marcada discontinuidad que existe en el espectro de sus fenotipos. La acti-

vidad de la enzima puede ser restaurada por complementación *in vivo* o *in vitro*, requiriéndose en este último caso tratamiento con 2-mercaptoetanol. La enzima obtenida por complementación es muy termolábil comparada con la de la cepa silvestre, y esta labilidad es mayor para el caso del producto de la complementación *in vitro* que para el de la enzima obtenida por complementación *in vivo*. Se discute el significado de este hallazgo en relación al mecanismo de complementación interalélica.

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DISCUSSION

Wolff: If the second scheme that you have presented (the one you left on the board) is correct, would you not expect that any two mutants that were separable on the genetic map would complement?

Reissig: No, really I do not think one knows what to expect. The diagram I drew is highly simplified and it has to be translated in terms of tertiary structure, with due regard to the nature of the active site, to allow us to decide when complementation will occur. Crick has discussed the general requirements for complementation in a recent paper.

Giles: Dr. Reissig, do you have a group of completely noncomplementing mutants which presumably represent nonsense mutants or something of the sort that would not interact perhaps because they do not make a complete polypeptide chain?

Reissig: We do. They are not very frequent; they are less than 10 percent of the total number of mutants.

Allende: Relative to the effectiveness of renaturation of the protein molecule in *in vivo* systems as compared to *in vitro* experiments, the results of Dr. Anfinsen might be pertinent. He studies the reoxidation of ribonuclease that has been denatured by reduction of the disulfide bonds to form SH derivatives and then finds that the reoxidation of the protein which regenerates the active enzyme is much faster in the presence of ribosomes and a soluble factor obtained from the liver supernatant fraction.

Reissig: That is a very interesting point.

Impaired Complementation Between Nonallelic Mutations in *Neurospora*[1]

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SUMMARY

Several approaches for the analysis of the control of gene function at the biochemical level have recently been developed in microorganisms. A new system is described for the genetic analysis of effects, generally associated with mutations induced in diploid organisms, in the haploid organism *Neurospora*. The system consists of a genetically marked 2-component heterokaryon (referred to as a dikaryon) that permits the recovery and analysis of both intragenic and extragenic mutations at specific loci. Mutations induced in the dikaryon at two closely linked loci (*ad-3A* and *ad-3B*) in the *ad-3* region with X-ray treatment may be grouped as follows: (*a*) *ad-3*^{R} mutations—reparable mutations that increase linearly with dose, and will grow as homokaryons on adenine-supplemented minimal medium; and (*b*) *ad-3*^{IR} mutations—irreparable mutations that increase as the square of the dose and will not grow as homokaryons even on complete medium. Nonallelic complementation between pairwise combinations of *ad-3*^{R} and *ad-3*^{IR} mutations at the *ad-3A* and *ad-3B* loci has been studied by determining the linear growth rates of forced dikaryons. Such analyses have led to the following general conclusions: (*a*) *ad-3A*^{R} + *ad-3B*^{R} combinations grow at wild-type growth rate; (*b*) *ad-3A*^{IR} + *ad-3B*^{R} or *ad-3A*^{R} + *ad-3B*^{IR} combinations may grow at less than wild-type growth rate; and (*c*) *ad-3A*^{IR} + *ad-3B*^{IR} combinations always grow at less than wild-type rate and may grow 10 times more slowly. The reduction in the growth rates of dikaryons involving *ad-3*^{IR} mutations has been interpreted as resulting from reduced functional activity of nonallelic loci in the immediately adjacent regions. Such behavior provides presumptive evidence for position effects in the *ad-3* region of *Neurospora*. The extent of genetic damage associated with the inactivation of the *ad-3A* or *ad-3B* locus in the *ad-3*^{IR} mutations can be estimated, and the extent of the functional inactivation of linked wild-type loci in the immediate vicinity of the *ad-3* region can be determined by means of homology tests with genetically marked strains containing linked biochemical or morphological markers. The implications of these findings are discussed in relation to proposed studies of the mechanism of position effects at the biochemical level.—Nat Cancer Inst Mongr 18: 33–52, 1965.

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

ONE OF THE most intriguing areas for genetic analysis is the study of the control of gene function. At one time it was generally assumed that the action of genes producing functional proteins was autonomous, but many recent investigations indicate that the primary action of structural genes is subject to various mechanisms of control. In the prokaryotes, Jacob and Monod's studies (*1*) on the β-galactosidase region in *Escherichia coli* and Ames and Hartman's studies (*2–4*) on the region affecting histidine biosynthesis in *Salmonella* have demonstrated a mechanism for the coordinate control of the action of linked genes affecting the same biosynthetic pathway by means of operator genes. Each of these regions constitutes a unit of coordinated transcription termed an operon (*1*). That such control mechanisms are not limited to the prokaryotes has been shown by Giles and his associates (*5, 6*), who have demonstrated the existence of an operon in the eukaryote *Neurospora*. These investigators have shown that the studies of Webber (*7–9*) and Ahmed (*6*) can be interpreted in terms of an operon, and they have proposed that the *hist-3* region consists of an operator gene and at least 3 genes controlling different steps in histidine biosynthesis. Other types of control mechanisms have undoubtedly evolved for the regulation of gene action in higher organisms along with the organization of the DNA into chromosomes of more complex structure and function, and studies on other eukaryotes have provided evidence for more complex types of interaction. Studies on the relations between genes in maize have shown that the action (and mutability) of a structural gene appears to be determined by the presence of controlling elements at or near the gene locus (*10*). In addition, in *Drosophila*, *Oenothera* [*see* review by Lewis (*11*)], and mice (*12–14*), complex effects on the function of genes have been observed when their associations with neighboring genes are changed as a consequence of certain types of chromosome rearrangement. This latter category of effects has been referred to as position-effect mutation, and may be either of the variegated or stable type. In brief, these examples show that the genes are not autonomous units and suggest that their primary activity can probably be regulated by various types of control mechanisms in all organisms.

Variegated-type position effects in *Drosophila* and mice are particularly interesting in that the mutational event affects not only a single gene, but all of those genes in close association with the sites of chromosome rearrangement. We know that the variegated-type position effects in *Drosophila* are associated with euchromatin and heterochromatin rearrangement and do not result from any change in the primary structure of those genes showing variegation. In both *Drosophila* and mice it has been possible to show 1) that normal function is restored to those genes showing variegation when they are removed from the site of the rearrangement either to their normal position by crossing-over or to a new euchromatic position by X-ray treatment, and 2) that wild-type genes will show variegation when they are moved from their normal position by crossing-over to the site of a rearrangement [*see* reviews by Lewis (*11*) and Russell and Bangham (*12*)].

Although many of the experiments in this general area are 30 to 40 years old, investigation of the mechanisms responsible for position effects is still one of the relatively unexplored areas of genetics. The rapid technical advances in the use of some of the "microorganisms" among the eukaryotes for genetic analysis in recent years make possible novel approaches to these fundamental problems. Since the DNA in the green algae and the fungi is organized into chromosomes in a manner similar to, if not identical with, that found in higher organisms, it should be possible to investigate the mechanism of variegation at the biochemical level.

In *Neurospora,* nonallelic mutations show full complementation, and heterokaryons between nonallelic biochemical markers grow at wild-type rate. Departures from this generality can usually be attributed to the unsuspected heterozygosity for factors affecting either heterokaryon formation or maintenance (*15–19*). Chromosome aberrations resulting in position effects, studied so extensively in higher organisms, are unknown in microorganisms. It is possible that this is because mutations with spreading effects are homokaryotic lethal in haploid organisms. In *Neurospora* the only mutations which have been studied extensively are those that can grow as homokaryons on supplemented minimal medium or complete medium. Such mutations have been classified as "reparable mutations," and they are reparable in the sense that the genetic alteration leads to a requirement that can be satisfied by (usually a single) biochemical supplementation of the basal minimal medium. Irreparable mutations are also known; these behave as recessive lethals even on complete medium (*20*), and it is generally assumed that these are point mutations of structural genes which produce nonsupplementable requirements, or point mutations of regulatory genes with nonsupplementable loss of function.

The recent development of a new procedure for forward-mutation experiments in *Neurospora* (*21*, *22*) makes it possible to induce, maintain, and analyze the same spectrum of genetic alterations at specific loci in a haploid organism as is found at specific loci in diploid organisms. This procedure makes use of a 2-component heterokaryon, and both homokaryotic viable (reparable mutations) and homokaryotic lethal (irreparable mutations) have been recovered (*21*). Other techniques have been developed for the identification of those mutations with effects more complex than the functional inactivation of a single genetic locus. Genetic analyses of the reparable and irreparable mutations induced at closely linked loci in the *ad-3* region of *Neurospora* have provided presumptive evidence for position effects in *Neurospora* and a means for the investigation of this phenomenon in microorganisms. The investigations reported here represent the first attempts toward the identification of mutations with spreading effects and the development of techniques and approaches for more critical genetic analysis.

MUTANT ISOLATION

The mutations for this analysis were induced at two different but closely linked loci on linkage group I (LGI) in the *ad-3* region of *Neurospora* designated *ad-3A* and *ad-3B* (*23*). Mutations at either of these loci are phenotypically identical; they accumulate purple pigment in the vacuoles of the mycelium and have a requirement for adenine. Biochemical analyses have shown that they affect the same or sequential steps in purine biosynthesis (*24*). Each component of the heterokaryon (referred to as a dikaryon) used in the forward-mutation experiments contains a series of biochemical markers, as shown in table 1. This dikaryon was designed so that any genetic alteration inactivating the *ad-3A* or *ad-3B* locus, or both, in component II in a heterokaryotic conidium, will result in the formation of a reddish-purple colony in adenine-supplemented medium. All other heterokaryotic conidia form colorless colonies, and because of the difference in the pigmentation between mutant and nonmutant colonies those colonies containing *ad-3* mutations can be recovered by a direct method (*22*, *25*). With this method the entire population of treated conidia are incubated, and each conidium surviving mutagenic treatment forms a microcolony about 2 mm in diameter. With an average of 10^6 colonies per incubation flask and forward-mutation rates ranging from 3 to 300 per 10^6 survivors (*26*), recovery of large numbers of mutants after treatment with specific mutagens is readily accomplished.

TABLE 1.—Genetic composition of each component of the dikaryon used in forward-mutation experiments*

Linkage group	Genotype of each component Component I (strain number 74-OR60-29A)	 Component II (strain number 74-OR31-16A)
I	*A hist-2 ad-3A ad-3B nic-2*	*A al-2*
III	*ad-2*	—
IV	—	*cot*
V	*inos*	—
VI	—	*pan-2*

*Genetic markers are as follows: *A*—mating type; *hist-2*—histidine-requiring; *ad-3A*, *ad-3B*, and *ad-2*—adenine-requiring; *nic-2*—niacin-requiring; *al-2*—albino mycelium and conidia; *cot*—temperature-sensitive morphological (slower growth the higher the temperature); *inos*—inositol-requiring; *pan*—pantothenate-requiring.

CHARACTERIZATION OF *ad-3* MUTATIONS

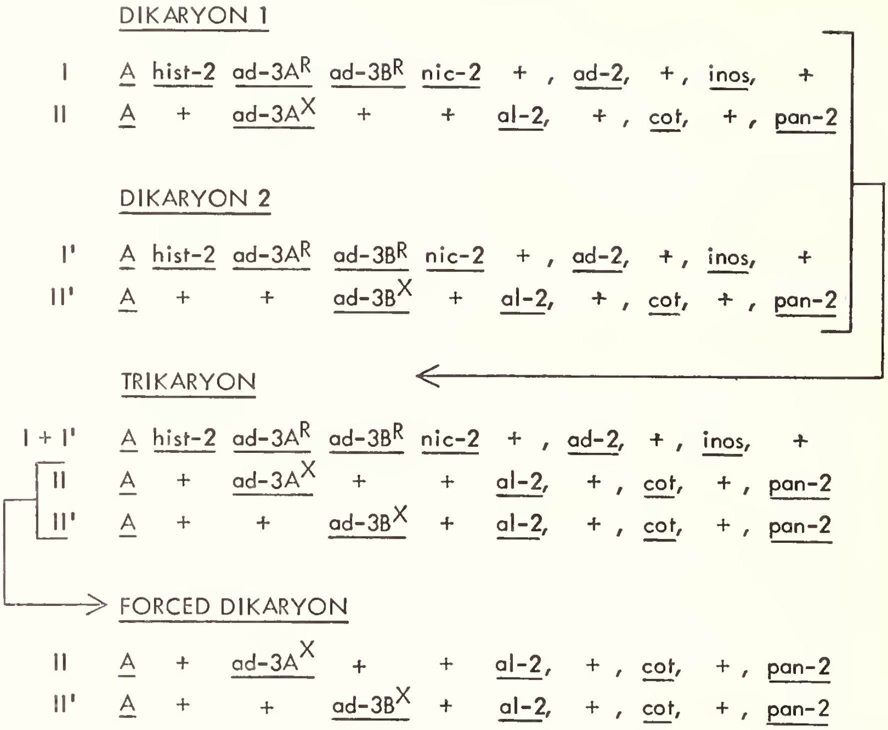
Genetic analysis of the mutations induced in a dikaryon after X-ray treatment showed that these mutations can be subdivided into two main classes: *reparable mutations* (*ad-3*^{R}) that will grow as homokaryons on adenine-supplemented medium, and *irreparable mutations* (*ad-3*^{IR}) that will not grow as homokaryons even on complete medium (*27*). The reparable mutations are always of genotype *ad-3A* or *ad-3B*, but the irreparable mutations can be either *ad-3A*, *ad-3B*, or *ad-3A ad-3B* double mutants. Genotypes are determined by a series of simple heterokaryon tests with 4 different tester strains of the following genotypes: 1) *ad-3A*, 2) *ad-3B*, 3) *hist-2 nic-2*, and 4) *ad-2 inos*. Since the markers in each of these 4 tester strains are found in component I of the dikaryon, such heterokaryon tests involve only the reaction of component II of the dikaryon with the testers.

INTERACTION BETWEEN *ad-3*^{R} AND *ad-3*^{IR} MUTATIONS IN FORCED DIKARYONS

The interaction between the reparable and irreparable mutations at these two loci was studied first by combining them in various pairwise combinations in forced dikaryons, and then determining their linear growth rates. This was accomplished by forcing heterokaryon formation between the dikaryons containing reparable or irreparable mutations at the *ad-3A* locus with other dikaryons containing reparable or irreparable mutations at the *ad-3B* locus to form tetrakaryons (really trikaryons, since 2 of the 4 components are identical in genotype), and then extracting the forced dikaryons with these nonallelic mutations in pairwise combination, as indicated in text-figure 1. When these forced dikaryons were isolated from the conidial platings of the trikaryons and single-colony isolates were transferred to the bottom of agar slants in test tubes, it was apparent that there were marked differences in the growth rates of different mutant combinations. The slow growth of the combinations of irreparable mutants was particularly noticeable, whereas those involving *ad-3*^{R} + *ad-3*^{IR} combinations or *ad-3*^{R} + *ad-3*$^{R'}$ combinations appeared to grow at wild-type rate.

It was impossible to obtain forced dikaryons for some of the combinations of irreparable mutations (*27*), and the significance of this observation will be discussed in a later section.

To obtain more precise data on the growth of these forced dikaryons, the linear growth rates were determined in horizontal growth tubes (*28*). Heavy suspensions of conidia from two single-colony isolates of each dikaryon (about 10^8 conidia/ml) were used to inoculate duplicate growth tubes containing pantothenate-supplemented medium. Each tube was marked daily for 7 to 30 days to record the total linear growth made in successive 24-hour periods. There was considerable variation in the linear growth rates of single-colony isolates from some of the dikaryons. Because the slower growth rates of some of the single-colony isolates can probably be attributed to markedly skewed nuclear ratios (or more stringent requirements for nuclear ratios approximating 1:1), a large number were isolated and tested. For the growth-tube experiments only the most rapidly growing single-colony isolates of each combination were used. Average growth rates were determined in the region of the growth tube distal to that point, either where the growth rate became constant or where the maximum growth rate was obtained. The linear growth rates


DIKARYON 1
I A hist-2 ad-3A^R ad-3B^R nic-2 + , ad-2, +, inos, +
II A + ad-3A^X + + al-2, + , cot, + , pan-2
DIKARYON 2
I' A hist-2 ad-3A^R ad-3B^R nic-2 + , ad-2, +, inos, +
II' A + + ad-3B^X + al-2, + , cot, + , pan-2
TRIKARYON
I + I' A hist-2 ad-3A^R ad-3B^R nic-2 + , ad-2, + , inos, +
II A + ad-3A^X + + al-2, + , cot, + , pan-2
II' A + + ad-3B^X + al-2, + , cot, + , pan-2
FORCED DIKARYON
II A + ad-3A^X + + al-2, + , cot, + , pan-2
II' A + + ad-3B^X + al-2, + , cot, + , pan-2

Text-figure 1.—Procedure for obtaining forced dikaryons between nonallelic reparable or irreparable mutations at the *ad-3A* locus ($ad\text{-}3A^X$) with reparable or irreparable mutations at the *ad-3B* locus ($ad\text{-}3B^X$) [modified from de Serres (*27*)].

of the pooled data from the analysis of these dikaryons are presented in table 2. The linear growth rate of strain 74-OR31-16A, component II of the dikaryon in which these mutations were induced, was 2.83 ± 0.05 mm per hour in the same experiment.

The linear growth rates of dikaryons involving $ad\text{-}3^R + ad\text{-}3^{IR}$ combinations can be best compared with those of the dikaryon between the two reparable mutants 11-1-11 + 11-1-1 (hereafter these mutant symbols will be abbreviated by using only the last number, *i.e.*, 11-1-11 + 11-1-1 = 11 + 1) used as a control. The data show that this dikaryon grows at the same rate as "wild-type" strain 74-OR31-16A. Whereas most of these dikaryons grow as rapidly as 11 + 1, the dikaryons involving the $ad\text{-}3A^{IR}$ mutants 13 and 122 and the $ad\text{-}3B^{IR}$ mutants 10, 121, and 123 show significant reductions in linear growth rate.

The most striking feature of these data is the marked reduction in linear growth rate found with all combinations of irreparable mutants as compared with that of the 2 reparable mutants 11 + 1. There is almost a tenfold difference between the growth rate of this combination and the growth rate of the dikaryon involving 122 + 121.

The marked variability in the growth of certain combinations of irreparable mutations is reflected in the unusually high confidence limits

TABLE 2.—Mean linear growth rates (mm/hr)* of pairwise combinations of *ad-3*^{R}† and *ad-3*^{IR}† mutations in forced dikaryons (on minimal medium + 2 μg per ml calcium pantothenate ~23° C)

Genotype and strain number		*ad-3B*^{IR}					*ad-3B*^{R} 11–1–1
		11–1–2	11–1–7	11–1–10	11–1–121	11–1–123	
ad-3A^{IR}	11–1–5	2. 26 ± 0. 03	2. 10 ± 0. 05	1. 70 ± 0. 02	1. 99 ± 0. 02	1. 73 ± 0. 06	2. 80 ± 0. 04
	11–1–6	2. 29 ± 0. 08	2. 19 ± 0. 03	1. 52 ± 0. 10	1. 79 ± 0. 08	1. 00 ± 0. 20	2. 83 ± 0. 05
	11–1–8	2. 18 ± 0. 04	1. 62 ± 0. 12	1. 58 ± 0. 04	1. 41 ± 0. 04	1. 94 ± 0. 13	2. 80 ± 0. 04
	11–1–13	2. 24 ± 0. 06	1. 56 ± 0. 31	—	—	1. 81 ± 0. 16	2. 77 ± 0. 04
	11–1–122	2. 31 ± 0. 06	1. 42 ± 0. 07	—	0. 32 ± 0. 02	1. 08 ± 0. 04	2. 58 ± 0. 14
ad-3A^{R}	11–1–11	2. 86 ± 0. 05	2. 80 ± 0. 07	2. 72 ± 0. 05	2. 59 ± 0. 18	2. 55 ± 0. 26	2. 91 ± 0. 08

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* Quadruplicate growth tubes for each dikaryon, 95 percent confidence limits given for each estimate.

† R = reparable recessive lethal mutation on minimal + adenine or complete medium; IR = irreparable recessive lethal mutation on minimal + adenine or complete medium; — = combination not viable as a dikaryon.

for the growth rates of such dikaryons as 13 + 7, 13 + 123, 6 + 10, 6 + 123, 8 + 123, 11 + 121, 11 + 123, and 122 + 1. The phenotype of these dikaryons is markedly different from those dikaryons involving different combinations of reparable mutations. The mycelial frontier in *ad-3*^{IR} dikaryons is erratic with both fast and slow growing sectors and the growth rates of single-colony isolates of the same dikaryon can vary markedly in the same 24-hour period. The contrast between the variegated phenotype of the dikaryons involving irreparable mutations and the regular concentric phenotype of dikaryons involving reparable mutations is particularly striking when they are inoculated on the surface of agar in the center of a petri plate.

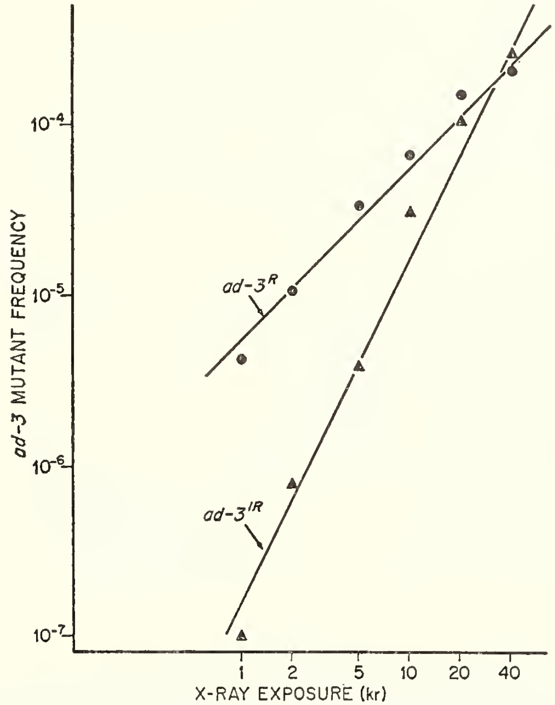
The normal growth of dikaryons involving an *ad-3A* and an *ad-3B* mutant on minimal medium should depend on the presence of a combined complement of the unaltered genetic material in the *ad-3* and immediately adjacent regions. The evidence from homology tests on *ad-3*^{R} and *ad-3*^{IR} mutations [de Serres (*27*) and unpublished data] indicates that the functional inactivation in *ad-3*^{R} mutants does not extend beyond the boundaries of the *ad-3A* or *ad-3B* cistron. Forced dikaryons between *ad-3B*^{R} and *ad-3A*^{R} mutants have a combined complement containing a normally functioning *ad-3A* and *ad-3B* locus, and these always grow at wild-type rate. Any spreading effects of the genetic alterations producing *ad-3A*^{IR} or *ad-3B*^{IR} mutations, however, would affect the activity of loci in the region between the *ad-3A* and *ad-3B* locus in addition to loci in the regions beyond, both to the right and to the left. Furthermore, the growth rates of various combinations of reparable and irreparable mutations in pairwise combinations should provide some measure of any reduction in functional activity. In some cases wild-type growth rate can be obtained with as little as 3 percent of the wild-type enzyme activity, as shown by Woodward, Partridge, and Giles (*29*) for revertants of *ad-4* mutants. If the same situation applies to the *ad-3A* or the *ad-3B* locus, then any observed reduction in growth rate could result from quite drastic alterations in the activity of either of these two loci. That the wild-type *ad-3A* locus shows less than normal function in certain *ad-3B*^{IR} mutants and that the wild-type *ad-3B* locus shows less than normal function in certain *ad-3A*^{IR} mutants are evident from the slower rates of growth of some of the *ad-3*^{R} + *ad-3*^{IR} combinations in table 2. The consequences of these spreading effects are enhanced in various *ad-3*^{IR} + *ad-3*$^{IR'}$ combinations when a partially inactivated wild-type *ad-3A* locus is combined with a partially inactivated wild-type *ad-3B* locus. Since both wild-type loci have reduced functional activity, the dikaryons between them show more striking reductions in growth rate.

NATURE OF THE GENETIC ALTERATIONS PRODUCING *ad-3*^{R} AND *ad-3*^{IR} MUTANTS

Mutations showing position effects in *Drosophila* are associated with such chromosome rearrangements as inversions and translocations [*see* review by Lewis (*11*)]. Such extragenic mutations result from chromosome breaks in two different places in the genome and should increase as the square of the dose with X rays. Point mutations or intragenic alterations result from molecular rearrangements (or alterations) within the gene, and should increase linearly with dose with X rays.

An attempt was made to determine the nature of the genetic alterations producing *ad-3*^{R} and *ad-3*^{IR} mutations by an analysis of the dose-response curves obtained after exposure of the dikaryon, shown in table 1, to X rays (*30*). In this analysis, the frequency of all *ad-3* mutants (or purple colonies) was found to increase as the 1.36 power of the dose. Genetic analysis of mutants showed that the *ad-3*^{R} mutants increase in direct proportion to dose, whereas the *ad-3*^{IR} mutants increase as the square of the dose (text-fig. 2). *Ad-3* mutations with more extensive functional inactivation affecting the *nic-2* locus some 3.0 map units distal were also sought in this experiment, and 2 additional classes of *ad-3*^{IR} mutants were found, the first of genotype (*ad-3B nic-2*)^{IR} and the second (*ad-3A ad-3B nic-2*)^{IR}.

This experiment provides direct evidence that *ad-3*^{IR} mutants result from 2-hit events, and it may be surmised that they result from such extragenic alterations as inversions, deletions, or translocations. It is of particular interest that *ad-3*^{IR} mutations are not produced in a com-



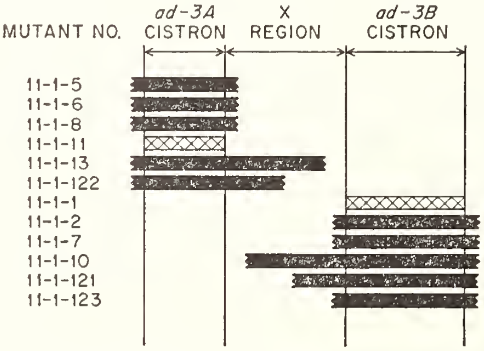
Text-figure 2.—Forward-mutation frequencies for *ad-3*^{R} and *ad-3*^{IR} mutations after exposure to 250 kv X rays [modified from Webber and de Serres (*30*)].

parable experiment in which the same dikaryon was treated with nitrous acid (Brockman, de Serres, and Barnett, in preparation). We may conclude from the nitrous acid experiment that *ad-3*^{IR} mutations result solely from extragenic alterations, and that they are not produced by an agent producing mutations predominantly by base-pair substitution in *Neurospora* [de Serres (*26*); de Serres, Brockman, Barnett, and Kølmark, in preparation].

SIGNIFICANCE OF THE APPARENT HOMOLOGY OF CERTAIN *ad-3A*^{IR} AND *ad-3B*^{IR} MUTATIONS

As mentioned in a previous section, it was not possible to isolate a forced dikaryon homozygous for the markers *cot*, *al-2*, *pan-2* from the trikaryons between certain *ad-3A*^{IR} + *ad-3B*^{IR} combinations: 13 + 10, 13 + 121, and 122 + 10. After several different experimental approaches failed to yield forced dikaryons between these combinations, we concluded that they must contain homologous or overlapping areas of functional inactivation (*27*). The simplest assumption was that the extensive functional inactivation associated with these mutations resulted from genetic damage located in the region between these two loci; therefore, these mutants have been represented as overlapping on a complementation map of the *ad-3* region (text-fig. 3).

The analysis of the induction of *ad-3*^{R} and *ad-3*^{IR} mutations with X rays indicates that *ad-3*^{R} mutants are not converted to *ad-3*^{IR} mutants with increasing X-ray exposure, but rather that each *ad-3*^{IR} mutant results from 2 hits, and that each of these hits is not detectable as an *ad-3* mutation by itself. Therefore, we have assumed that they must occur on either side of the boundaries of either the *ad-3A* or *ad-3B* locus and result in deletion of the genetic material between them. The implication of the



TEXT-FIGURE 3.—Complementation map of X-ray-induced reparable and irreparable recessive lethal mutations in the *ad-3* region showing the extent and type of functional damage in individual mutations. *Solid bars* = irreparable recessive lethal mutations; *irregular ends* indicate unknown limits; *crosshatched bars* = reparable recessive lethal mutations [adapted from de Serres (*27*)].

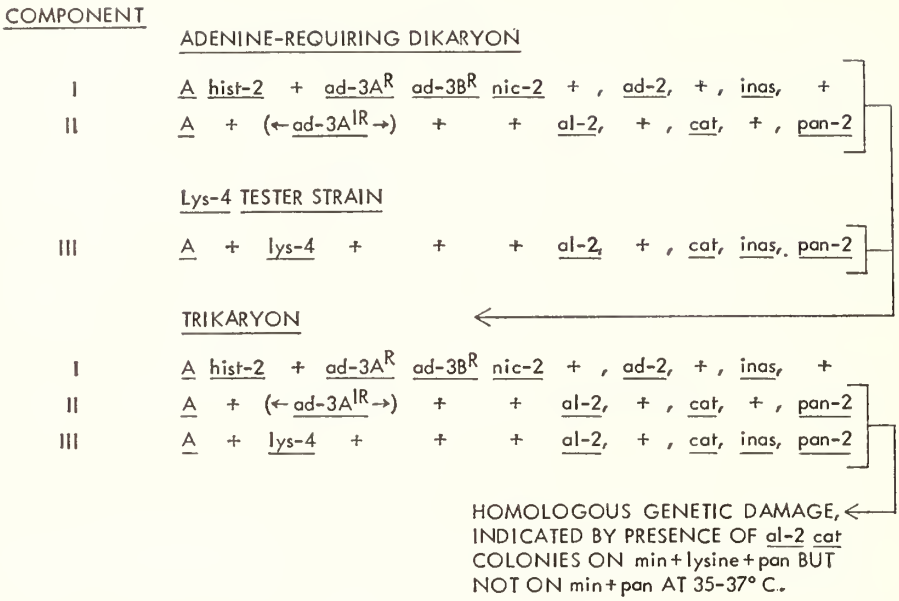
homology between certain nonallelic *ad-3*^{IR} mutations is that they have genetic damage in common located outside of the *ad-3A* or *ad-3B* locus, and this interpretation supports the conclusions drawn from the X-ray induction studies.

In summary, with genetic analysis by means of homology tests, it seems possible to determine *the extent of genetic damage* in individual *ad-3*^{IR} mutations by the failure of various combinations to show complementation, and *the extent of the functional inactivation* (or spreading effect) by the growth rates of the *ad-3*^{IR} mutations in combination with reparable mutants at other loci in the immediately adjacent regions. The genetic damage need not be precisely homologous when nonallelic irreparable mutants fail to complement. However, it is reasonable to assume that *at a minimum* they each possess some genetic damage affecting the same cistron.

GENETIC ANALYSES INVOLVING CLOSELY LINKED MARKERS IN THE IMMEDIATELY ADJACENT REGIONS

Homology tests between *ad-3*^{IR} mutations and reparable mutants at other closely linked loci represent one of the most critical tests in the analysis of *ad-3*^{IR} mutants. If the apparent reduction of functional activity of loci in the vicinity of an irreparable mutation is indeed analogous to the spreading effects associated with variegated-type position effects in *Drosophila*, then the intensity of the effect should decrease with increasing map distance and not be present in tests with unlinked markers (unless they have been brought into new associations as a result of chromosome translocation). Many closely linked morphological and biochemical markers are known both to the left and to the right of the *ad-3* region [Perkins (*31*); de Serres, unpublished data]. Homology tests with such markers require strains heterokaryon-compatible with mutants induced in a 74A genetic background in addition to forcing biochemical and identifying morphological markers, as shown in text-figure 4. The same general procedure shown for LGI markers is used in complementation tests with unlinked markers. In order for such tests to be unequivocal, it is important that components II and III of the trikaryon are heterozygous for at least one marker in addition to the one being tested and homozygous for the markers *al-2*, *cot*, and *pan-2*. Because components II and III are always heterozygous for the *inos* marker, it is possible to look for *cot* colonies both on supplemented and unsupplemented media (with reference to the marker at the locus being tested). This is to ensure that this particular combination of genomes can make a *cot* dikaryon, and that the absence of *cot* colonies cannot occur for other reasons (reversion of the *cot* marker, or loss by somatic recombination, etc., in either component II or III).

For example, to perform homology tests with a reparable mutant at the *lys-4* locus some 1.3 map units to the left (de Serres, unpublished data),


COMPONENT
ADENINE-REQUIRING DIKARYON
I
A hist-2 + ad-3A^R ad-3B^R nic-2 + , ad-2, + , inos, +
II
A + (←ad-3A^{IR}→) + + al-2, + , cat, + , pan-2
Lys-4 TESTER STRAIN
III
A + lys-4 + + + al-2, + , cat, inos, pan-2
TRIKARYON
I
A hist-2 + ad-3A^R ad-3B^R nic-2 + , ad-2, + , inos, +
II
A + (←ad-3A^{IR}→) + + al-2, + , cat, + , pan-2
III
A + lys-4 + + + al-2, + , cat, inos, pan-2
HOMOLOGOUS GENETIC DAMAGE, INDICATED BY PRESENCE OF al-2 cat COLONIES ON min+lysine+pan BUT NOT ON min+pan AT 35-37° C.

TEXT-FIGURE 4.—Procedure to determine the extent of genetic damage and functional inactivation in irreparable *ad-3* mutations with the use of genetic markers at loci in the immediately adjacent genetic regions. The procedure is illustrated for tests with a marker at the *lys-4* locus (lysine-requiring).

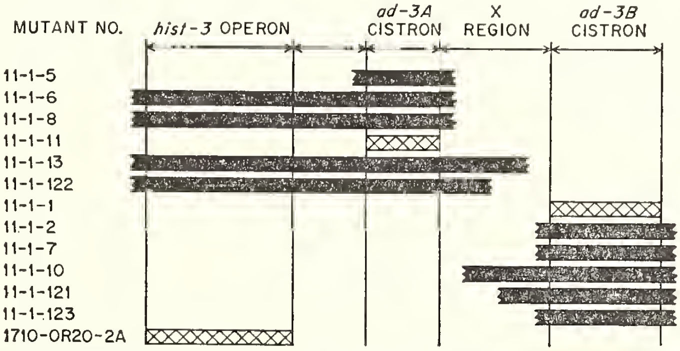
a trikaryon is made between the dikaryon containing an *ad-3A*^{IR} mutation and the appropriately marked strain containing the *lys-4* marker on minimal medium. The trikaryon is then plated on minimal medium supplemented with pan (calcium pantothenate) and minimal medium supplemented with lysine and pan, and the plates are incubated at 35 to 37° C. Neither component II nor component III can form *cot* colonies on either of these media, but the dikaryon between them will form *cot* colonies only on the doubly supplemented medium if the genetic damage in the *ad-3A*^{IR} mutation covers the *lys-4* locus, and on both media if it does not. If the genetic damage in the *ad-3A*^{IR} mutation is not homologous to that found in the *lys-4* mutant, then the functional activity of the wild-type *lys-4* locus can be determined by measuring the linear growth rate of the *cot* colonies at 20° C.

Appropriately marked strains for such tests with mutants at the *hist-2*, *lys-4*, *hist-3* loci to the left and the *nic-2* locus to the right have been synthesized only recently, and only preliminary data are currently available. The results of homology tests based on the appearance of *cot* colonies from the different trikaryons are shown in text-figure 5. The genetic damage in none of the *ad-3B*^{IR} mutants analyzed was homologous to that in the *nic-2* marker, but the genetic damage in the *ad-3A*^{IR} mutants 6, 8, 13, and 122 was homologous to that found in the *hist-3* marker. No information is available on the linear growth rates.

Although incomplete, these preliminary results show quite clearly that the genetic damage in *ad-3A*^{IR} mutations can extend to the left and/or the right of the *ad-3A* locus.

CORRELATIONS BETWEEN COMPLEMENTATION MAP POSITION AND THE GROWTH RATES OF *ad-3*^{IR} MUTANTS IN FORCED DIKARYONS

There is a correlation between the position and extent of individual *ad-3*^{IR} mutants on the complementation map (text-fig. 5) and the rates of growth of *ad-3*^{R} + *ad-3*^{IR} combinations in forced dikaryons (table 2). Mutants 13 and 122 show more extensive homology with other mutants than 5, 6, and 8; 13 and 122 show much slower growth rates in the forced dikaryons. The same type of correlation exists among the *ad-3B*^{IR} mutants. Mutants 10 and 121 show more extensive homology than mutant 2 or 7, and show much slower rates of growth for heterokaryons with either reparable or irreparable *ad-3A* mutants. The correlation is by no means complete, since 123 is indistinguishable from 2 and 7 on the basis of present homology tests. It is possible that any correlation between the total extent of genetic damage and the intensity of the spreading effect at a particular linked genetic locus is obscured because individual *ad-3B* mutations have not been mapped on the right. By mapping these mutants more precisely it may be possible to determine whether the various types of interaction observed in the forced dikaryons are due to the position, type, or extent of the genetic damage in each of the two components. The effects associated with interstitial deletions might be quite different from those associated with chromosome translocations.



TEXT-FIGURE 5.—Complementation map of X-ray-induced reparable and irreparable recessive lethal mutations in the *ad-3* region showing the extent and type of functional damage in individual *ad-3A*^{IR} mutations in the regions to the left and to the right of the *ad-3A* cistron. *Solid bars* = irreparable recessive lethal mutations; *irregular ends* indicate unknown limits; *crosshatched bars* = reparable recessive lethal mutations. (Strain 1710-0R20-2A is a reparable, complementing *hist-3* mutant.)

DETECTION OF *ad-3*^{IR} MUTATIONS WITH MARKED SPREADING EFFECTS ON LOCI IN THE IMMEDIATELY ADJACENT REGIONS

The rationale of the approach used to screen large numbers of *ad-3*^{IR} mutations for those with the most extreme spreading effects is that such mutations should show a marked delay in the time of the appearance of a positive response in the heterokaryon tests used to determine genotype. *Ad-3A*^{IR} or *ad-3B*^{IR} mutations with extreme spreading effects will appear to be *ad-3A ad-3B* double mutants at the times the tests are normally scored (1 or 2 days), and positive reactions with the *ad-3A* or *ad-3B* tester will occur at some later time. Heterokaryon tests were made on 34 X-ray-induced *ad-3A*^{IR} and 68 X-ray-induced *ad-3B*^{IR} mutations to screen for such mutations, and we found that the reaction of 2 of the *ad-3B*^{IR} mutations with the *ad-3A*^{R} tester was delayed until at least 5 to 7 days after inoculation (Webber and de Serres, unpublished data). It is not likely that these delayed responses are the result of allelic complementation between different types of damage in the *ad-3A* locus, since extensive heterokaryon tests on *ad-3A* mutants of different mutagenic origin did not show allelic complementation (de Serres, unpublished data). Therefore we have concluded that these 2 *ad-3*^{IR} mutations are *ad-3B*^{IR} mutations and not (*ad-3A ad-3B*)^{IR} mutations. Homology tests between these 2 *ad-3B*^{IR} mutations and 16 of the *ad-3A*^{IR} mutations recovered from this same experiment (de Serres, unpublished data) have shown homology for all pairwise combinations tested. This is interpreted as indicating that the genetic damage in these *ad-3B*^{IR} mutants covers the entire region between the *ad-3A* and *ad-3B* loci, so that even those *ad-3A*^{IR} mutations that have resulted from chromosome breaks in close proximity to the *ad-3A* locus will show homology. This type of correlation suggests that the spreading effects associated with *ad-3*^{IR} mutations show a gradient in the intensity of their effects in *Neurospora*, as in *Drosophila* and mice. These additional data provide even more striking evidence that the functional activity of the wild-type *ad-3A* locus in *ad-3B*^{IR} mutants depends on proximity to the site of the chromosome damage in the *ad-3B*^{IR} mutant.

NEW APPROACHES FOR DETERMINING THE MECHANISM OF THE SPREADING EFFECTS ASSOCIATED WITH IRREPARABLE *ad-3* MUTATIONS

The influence of the spreading effects associated with *ad-3*^{IR} mutations on nonallelic complementation between them and reparable mutants at closely linked loci may well depend on whether the reparable mutants show allelic complementation. One might well ask whether the growth rate of such dikaryons is limited by the activity of the wild-type locus (in component II) of the linked reparable mutant being tested (in component

III) (*see* text-fig. 3) or whether the growth of the dikaryon is dependent on the activity of an interaction product. The type of interaction that can take place between the gene products specified by homologous loci in each component of these dikaryons may depend on whether allelic complementation is restricted to protein-protein interaction (*32*, *33*) or whether it can take place at other levels of organization (*34*).

The *ad-3A* locus is ideally situated for more detailed analysis of the mechanism of the spreading effects associated with certain *ad-3A*^{IR} mutations. It is in close proximity on the left (about 0.3–0.5 map units) to the *hist-3* operon (*5*) and closely linked on the right (about 0.1–0.3 map units) to the *ad-3B* locus. Mutants in the *hist-3* operon show allelic complementation, and the complementation map consists of at least 14 complementation units or complons (*8*). Mutants at the *ad-3B* locus also show allelic complementation, and the complementation map consists of at least 17 complons [de Serres (*26*) and unpublished]. A wide variety of complementing mutants with restricted complon coverage are available in both sets of mutants, so that it will be possible to perform tests for nonallelic complementation between various *ad-3A*^{IR} mutations with complementing mutants on different parts of each complementation map. From the work on *Drosophila* and mice there should be a gradient in the spreading effect across the *ad-3B* locus or the *hist-3* operon, so that the strength of the response obtained with different complementing mutants in tests for nonallelic complementation may depend on their location on the complementation map.

Any noteworthy effect on nonallelic complementation observed with the *hist-3* testers can be correlated with changes in biochemical activity. That part of the *hist-3* operon adjacent to the *ad-3* region is believed to be that controlling histidinol dehydrogenase activity (*9*), with the other two biochemical activities located further to the left. A comparison of the histidinol dehydrogenase activity 1) of dikaryons between *hist-3* testers lacking this activity in combination with *ad-3A*^{IR} mutations with 2) that of dikaryons between *hist-3* testers possessing this activity should provide information for the interpretation of the mechanism of the spreading effect phenomenon. We have no data from such trials as yet, since critical experiments require the use of genetically marked strains of *hist-3* mutants that are still in preparation. Nevertheless, this approach should provide significant information on whether 1) weak nonallelic complementation between *ad-3A*^{IR} and *hist-3* mutants can be attributed to a reduction in histidinol dehydrogenase activity, and 2) whether normal nonallelic complementation can be restored by providing a source of this enzyme activity in the other component of the dikaryon.

CONCLUSIONS

1. A new system is described for the genetic analysis of position effects (generally associated with mutations induced in diploid organisms) in the haploid organism *Neurospora*.

2. The system consists of a genetically marked 2-component heterokaryon (referred to as a dikaryon) that permits the recovery and analysis of both intragenic and extragenic mutations at specific loci.
3. Mutations induced in the dikaryon at two closely linked loci (*ad-3A* and *ad-3B*) in the *ad-3* region with X-ray treatment may be grouped as follows:
    - (*a*) *ad-3*^{R} mutations—reparable mutations that increase linearly with dose and will grow as homokaryons on adenine-supplemented minimal medium.
    - (*b*) *ad-3*^{IR} mutations—irreparable mutations that increase as the square of the dose and will not grow as homokaryons even on complete medium.
4. Nonallelic complementation between pairwise combinations of *ad-3*^{R} and *ad-3*^{IR} mutations at the *ad-3A* and *ad-3B* loci has been studied by determining the linear growth rates of forced dikaryons. Such analyses have led to the following general conclusions:
    - (*a*) *ad-3A*^{R} + *ad-3B*^{R} combinations grow at wild-type growth rate.
    - (*b*) *ad-3A*^{IR} + *ad-3B*^{R} or *ad-3A*^{R} + *ad-3B*^{IR} combinations may grow at less than wild-type growth rate.
    - (*c*) *ad-3A*^{IR} + *ad-3B*^{IR} combinations always grow at less than wild-type rate and may grow 10 times more slowly.
5. The reduction in the growth rates of dikaryons involving *ad-3*^{IR} mutations has been interpreted as a result of reduced functional activity of nonallelic loci in the immediately adjacent regions. Such behavior provides presumptive evidence for position effects in the *ad-3* region of *Neurospora.*
6. The extent of genetic damage associated with the inactivation of the *ad-3A* or *ad-3B* locus in the *ad-3*^{IR} mutations can be estimated, and the extent of the functional inactivation of linked wild-type loci in the immediate vicinity of the *ad-3* region can be determined by means of homology tests with genetically marked strains containing linked biochemical or morphological markers.
7. The implications of these findings are discussed in relation to proposed studies of the mechanism of position effects at the biochemical level.

RESUMEN

Algunos nuevos métodos de análisis del control de la función del gene al nivel bioquímico han sido recientemente desarrolladas en micro-organismos. Un nuevo sistema es descripto aquí para el análisis genético de los efectos, generalmente asociados con mutaciones inducidas por organismos diploides, en el organismo haploide Neurospora.

El sistema consiste en un heterokaryon genéticamente marcado de dos componentes (llamado dikaryon) que permite la recuperación y el análisis de las mutaciones intragénicas y extragénicas en loci específicos. Las mutaciones inducidas en el dikaryon en dos loci estrechamente unidos (*ad–3A* y *ad–3B*) en la región *ad–3* con un tratamiento de Rayos X pueden ser enumeradas de la forma siguiente: a (mutaciones *ad–3*^{R}—mutaciones reparables que aumentan linealmente con la dosis; ésta crecerá como homokaryones en un medio mínimo suplementado con adenina; y b) mutaciones

ad–3$^{\mathrm{IR}}$ – mutaciones irreparables que aumentan con el cuadrado de la dosis y que no crecerán como homokaryones aunque se hallen en un medio completo.

La complementación nonalélica entre combinaciones pares de mutaciones *ad–3*$^{\mathrm{R}}$ y *ad–3*$^{\mathrm{IR}}$ y las de *ad–3A* y *ad–3B* ha sido estudiada determinándose los grados de crecimiento linear de los dikaryones forzados.

Tales análisis han llevado a las siguientes conclusiones: a) las combinaciones ad-*3*A$^{\mathrm{R}}$ + ad-*3*B$^{\mathrm{R}}$ muestran un tipo de crecimiento muy rápido semejante al tipo salvaje; b) las combinaciones ad-*3*A$^{\mathrm{IR}}$ + ad-*3*B$^{\mathrm{R}}$ o ad-*3*A$^{\mathrm{R}}$ + ad-*3*B$^{\mathrm{IR}}$ pueden crecer en un ritmo menor de crecimiento menor que el tipo salvaje y c) las combinaciones ad–*3*A$^{\mathrm{IR}}$ + ad–*3*B$^{\mathrm{IR}}$ siempre crecen a un ritmo menor que el tipo salvaje has 10 veces menos.

La reducción en los niveles de crecimiento de los dikaryones que envuelven mutaciones ad-*3*$^{\mathrm{IR}}$ ha sido interpretada como resultante de una actividad functional reducida de loci non-aleli en las regiones inmediatamente adyacentes. Dicho comportamiento provce evidencia presuntiva de efectos de posición de tipo variegado en la región ad-*3* de la *Neurospora.* Puede estimarse, la extensión del perjuicio genético asociado con la inactivación de los locus ad-*3*A o ad-*3*B en las mutaciones *ad-3* $^{\mathrm{IR}}$y se puede determinar la extensión de la inactivación funcional de los loci salvaje ligados en la vecindad inmediata de la región *ad-3ã* por medio de pruebas de homología con cepas genéticamente marcadas con marcadores ligados bioquímicos o morfológicos. Las implicancias de estos hallazgos son discutidas con relación a los estudios propuestos del mecanismo de los efectos de posición del tipo variegado en un nivel bioquímico.

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DISCUSSION

Giles: I would like to ask Dr. de Serres whether there are any indications of mutants covering both the A and B regions completely.

de Serres: There are many mutants that test as AB doubles. When these are tested with markers at the *hist-2*, *lys–4*, or *hist-3* locus, it appears that many result from chromosome deletions including not only the *ad-3A ad-3B* loci, but also other loci in the immediately adjacent regions. The most important point in the whole discussion is that we have found no reparable mutants which are AB doubles. If the reparable A mutants occur at a frequency of about 1 per 10^6 survivors and the reparable B mutants occur at about 3 per 10^6 survivors, we would expect that the frequency of AB doubles would be 3 per 10^{12} survivors. We have examined several hundred mutants and have found none that test as reparable AB doubles. Furthermore, when you analyze the spectrum of adenine-3 mutations induced by X rays, AB double mutants increase as the square of the dose, which supports the hypothesis that they result from such 2 -hit events as chromosome deletion.

Reissig: When you get these extensive deletions, have you attempted to grow them on a supplemented media? What are the nutritional requirements?

de Serres: The point that I should have made more strongly is that the irreparable mutants do not grow as homokaryons on any medium. In other words, the reparable mutants will grow as homokaryons on an adenine-supplemented medium, but the irreparable mutants will not grow on any medium. All lines of evidence support the hypothesis that they result from chromosome deletions. Furthermore we have assumed that, since these mutations will not show complementation with markers to the left and to the right of the *ad-3* region, the wild-type loci of these markers are not present in the irreparable mutant being tested. We cannot get direct evidence by intercrossing, because the crosses of these mutants are sterile. Once our screening program is complete and we have large numbers of mutants that appear to result from deficiencies and others that appear to result from chromosome translocation, we should be able to differentiate one from the other by studying their X-ray-induced reversibility. The deficiency mutants should not revert, whereas at least some of those that might be due to position effects as a result of chromosome rearrangement should revert after X-ray treatment.

Schultz: I am somewhat disturbed about a question of nomenclature. In the case of the "spreading effects," one deals not with the selection of a mutant phenotype but with an analysis consequent on the accurate diagnosis of the point of breakage in the chromosome on which the genetic effect is observed. Now, in your case, I cannot see how you can possibly determine that. You have a selective technique for detecting the mutants and you have associated other differences with the selected types. Now, these may be differences that depend on the size of the deficiences or on genetic inactivation, whichever you would decide to call it. But it would seem to me that it would be incautious at the moment to use "spreading effect" for the kind of phenomenon that you are describing.

de Serres: It may be incautious, to use the same words, but I think the term "spreading effect" vividly describes the type of interaction that we have found in our tests on the irreparable mutants. There is something very different going on in our tests on the reparable mutants than there is on our tests on the irreparable mutants. I do not see why the term "spreading effect" does not apply to our tests with mutants of *Neurospora* as well as it applies in *Drosophila* or mice. You might ask the question: "What would variegated-type position effect look like in an organism like *Neurospora*, where you have a gene that is operative in some nuclei but not in others in a common mycelium?"

Schultz: I think we have a number of problems here. In the first place, when the Demerec group made a careful analysis of the sizes of deficiencies at a specific locus in *Drosophila*, there was a modal frequency of fairly short deficiencies and besides this there were larger and smaller ones to either side of the locus of the selected phenotype. Thus, there is no reason to infer that the locus for which you are selecting when you get these deficiences marks the boundary of the deficiency, and this is what you are assuming when you suppose that the wild-type genes at the left of the adenine locus need not be affected. You are chopping out a piece of chromosome and there is no *a priori* mode of telling where the exact boundaries of the piece are. This is a real question and so it seems to me that you must keep this dissociated from the other problem of variegation and spreading.

de Serres: To the contrary, I believe that the homology tests show the extent of genetic damage rather precisely and that the growth rates of the forced dikaryons (when they occur) constitute some measure of the extent of the functional inactivation. The whole point of measuring the growth rates of dikaryons involving irreparable mutants is to measure the functional activity of loci that have not been deleted.

Genetic Aspects of Colicinogeny [1]

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SUMMARY

The genetic behavior of colicinogenic factors E_1, E_2, I, V, and B in *Escherichia coli* K12 strains was studied. From analysis of crosses involving different Hfr and F+ strains as donors it follows that: 1) Each colicinogenic factor is transferred with similar frequencies whatever the type of donor bacteria involved. 2) The colicinogenic factors are not linked with chromosomal markers. 3) The noncolicinogenic character of the donor is never transmitted to recombinants. 4) Zygotes that have received the colicinogenic factor transmit it to all daughter cells. These results lead to the conclusion that colicinogenic factors are extrachromosomal in *E. coli* K12. Besides, the *col V–col I* factor possesses some properties characteristic of fertility factors since F− cells acquiring *col V–col I* factor become sensitive to phage *MS2* and transfer the (*col V–col I*) character at the same efficiency as F+ or Hfr strains. An interference with fertility is also manifested by (*col V–col I*) factor with different Hfr and F+ strains, since its presence in these strains drastically reduces the frequency of recombinant formation. Acquisition by an F−*lac*+ strain of the *col V–col I* factor was accompanied by loss of the F− *lac* factor, which suggests that entrance of *col V–col I* factor inhibits in some way the multiplication of the F− *lac* factor. These facts are discussed in connection with a possible interrelationship among colicinogenic, multiple drug resistance, and fertility factors.—Nat Cancer Inst Monogr 18: 53–64, 1965.

COLICINS are antibiotic substances produced by certain strains of *Enterobacteriaceae* which have bactericidal action on other strains of the same family. Their synthesis is controlled by hereditary factors known as colicinogenic factors. Colicinogeny can be transmitted among strains of the family *Enterobacteriaceae* either by cell contact or by transduction.

Studies on the transmission of colicinogenic factor E_1 (*col* E_1)—controlling the synthesis of colicin E_1—in crosses between F+ and F− strains of *Escherichia coli* K12 led Fredericq and Betz-Bareau (*1*) to conclude the cytoplasmic nature of this factor. The behavior of *col* E_1

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Functions, Buenos Aires, Argentina, November 30 to December 4, 1964.

was also studied by Alfoldi, Jacob, Wollman, and Mazé (*2*) in crosses involving Hfr donors, which transfer the chromosome at high frequency. Their results suggested that this factor, like temperate phages or fertility factors, might possess the properties assigned to episomal particles, that is, particles capable of occupying in the cell two different states: the integrated state on the chromosome and the autonomous state in the cytoplasm.

In 1960, while in Buenos Aires, Dr. E. L. Wollman suggested to our group we had formed with Dr. J. Puig the extension of these studies. Thanks to the continuous help and encouragement of Dr. Wollman, we carried out the study of the behavior of other colicinogenic factors.

In this paper, we will attempt to present the main results of our work on the genetics of colicinogenic factors E_1, E_2, I, V, and B.

MATERIALS AND METHODS

Each colicinogenic factor studied was transferred by mixed culture or by transduction to Hfr, F+, and F− strains of *E. coli* K12. Crosses between colicinogenic (*col*+) donors and noncolicinogenic (*col*−) recipients and the reciprocal ones, *col*− donor × *col*+ recipient, were performed, as well as the control crosses, *col*+ × *col*+ and *col*− × *col*−. All strains used in crosses were made resistant to the colicin involved in each cross, to avoid distortion of results due to the lethal action of the colicin on sensitive strains. The strains employed as donors and recipients in these crosses, as well as the original colicinogenic strains from which the colicinogenic factors were transferred, are listed in table 1.

Recombinants receiving the ability to synthesize an amino acid or vitamin from the donor parent and the streptomycin-resistant character

TABLE 1.—Bacterial strains

Strain	Sexual type	Characteristics*
Escherichia coli K12		
Hfr H	Hfr	$thi^{-}\ str^{s}$
Hfr P10	Hfr	$thr^{-}\ leu^{-}\ thi^{-}\ str^{s}$
Hfr P4X	Hfr	$met^{-}\ str^{s}$
Hfr AT IIIA	Hfr	Prototrophic str^{s}
112	F+	$cys^{-}\ his^{-}\ str^{s}$
W3101 F−*gal*+	F′	Prototrophic str^{s}
200 PS F− *lac*+	F′	$thi^{-}\ str^{s}$
PA 309	F−	$thr^{-}\ leu^{-}\ thi^{-}\ his^{-}\ try^{-}\ arg^{-}\ str^{r}$
C 600	F−	$thr^{-}\ leu^{-}\ thi^{-}\ lac^{-}\ str^{s}$ or str^{r}
W 3876	F−♀3	$lac^{-}\ mal^{-}\ str^{r}$
Original colicinogenic strains		
E. coli K12		
12–94		$met^{-}\ str^{r}\ (col\ V\text{–}col\ I)+$
12–317		$cys^{-}\ his^{-}\ str^{r}\ col\ E_2^{+}$
E. coli CA 53		Prototrophic $str^{r}\ col\ I^{+}$

**Abbreviations:* Requirement for *thr:* threonine; *leu:* leucine; *try:* trytophan; *his:* histidine; *arg:* arginine; *met:* methionine; *cys:* cysteine; *thi:* vitamin B_1; str^{s}: sensitivity to streptomycin; str^{r}: resistance to streptomycin; *lac:* fermentation of lactose; *mal:* fermentation of maltose; *gal:* fermentation of galactose.

from the recipient were selected on minimal medium supplemented with streptomycin. Transfer of *col*+ was measured on eosin methylene blue plates with streptomycin and transfer of *col*+ in nutrient agar plates with streptomycin, where only F— cells form colonies, the *col*+ character of these colonies being determined by the halos formed on a sensitive indicator strain.

The frequency of recombinants and transfer of *lac*+ or *col*+ is expressed as the ratio of the number of recombinants, *lac*+ str^r or *col*+ str^r cells, respectively, to 100 initial bacteria of the minority parental strain. The frequency of recombinants and of transfer of *col*+ was measured at 90 or 120 minutes after the onset of mating. Further details on the conditions of mating are given in the original papers (*3*, *4*).

RESULTS AND DISCUSSION

1. Genetic Behavior of Colicinogenic Factors

In crosses between colicinogenic donors and noncolicinogenic recipients, performed in the proportion of 1 donor to 20 recipient cells, the frequency of transfer of each colicinogenic factor involved was measured. As shown in table 2, each colicinogenic factor is transferred with similar frequencies whatever the type of donor cells involved (F+ or Hfr).

It may also be seen that different colicinogenic factors are transferred with quite different efficiencies. Thus, *col V* and *col* E_1 are transferred from F+ and Hfr cells with maximum efficiency, *col B* with intermediate frequency, and *col I* and *col* E_2, with low frequency.

The line of entry of colicinogenic factors V, E_1, and B was measured in matings interrupted by use of a Waring blendor. In this way it was established that these factors begin to appear among recipient cells within the first 10 minutes after the onset of mating, whatever the type of donor cell involved.

Different types of recombinants from crosses indicated in table 2 were selected, purified, and their *col*+ or *col*— character determined. As

TABLE 2.—Percent transfer of colicinogenic factors in crosses between ♂ str^s *col*+ × ♀ PA 309 str^r *col*—

Donor strain	Colicinogenic factor				
	V	E_1	B	E_2	I
F+	1000	600	14	1. 3*	0. 5*
Hfr H	850	780	29	0. 3	0. 1*
Hfr P4X	480	600	50	1. 3	—
Hfr P10	—	1000	11	3. 0	0. 1*
Hfr AT IIIA	2100	900	—	—	—

*These crosses were performed in the proportion of 20 donors to 1 recipient cell. All other crosses were in the proportion of 1 donor to 20 recipient cells.

TABLE 3.—Percentage of colicinogeny among different types of recombinants of crosses between Hfr *str*^{s} *col*+ and F− PA 309 *str*^{r} *col*−

Donor strains	Colicinogenic factors	Recombinants*			
		thr + *leu* + *str*^{r}	*try* + *str*^{r}	*his* + *str*^{r}	*arg* + *str*^{r}
Hfr H	V	79	91	100	100
	E$_1$	65	67	64	68
	E$_2$	0	0	0	2
	B	48	80	100	100
		thr + *leu* + *str*^{r}	*arg* + *str*^{r}	*his* + *str*^{r}	*try* + *str*^{r}
Hfr P4X	V	80	84	100	100
	E$_1$	57	59	75	68
	E$_2$	0	2	1	3
		arg + *str*^{r}	*his* + *str*^{r}	*try* + *str*^{r}	—
Hfr P10	E$_1$	58	57	73	
	E$_2$	0.3	0	1.5	
	B	35	90	98	
		his + *str*^{r}	*try* + *str*^{r}	*thr* + *leu* + *str*^{r}	*arg* + *str*^{r}
Hfr AT IIIA	V	92	98	100	100
	E$_1$	74	86	79	77

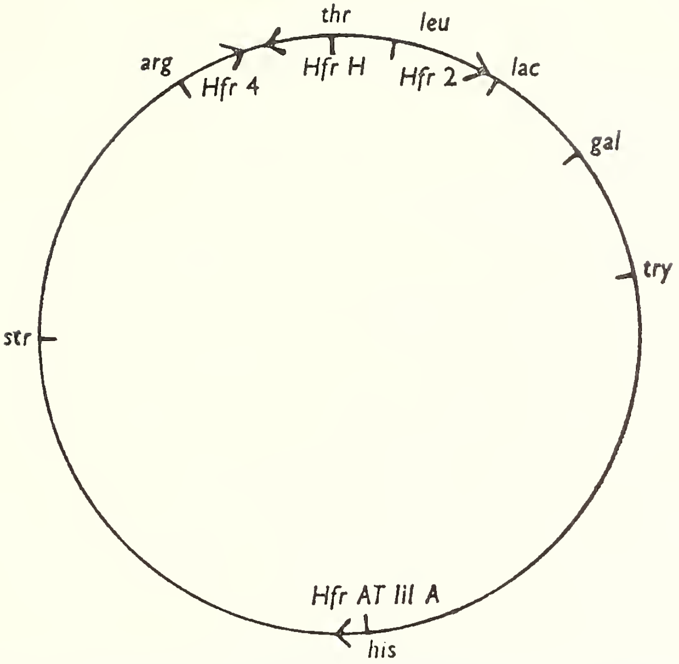
*For abbreviations, *see* footnote table 1.

shown in table 3, each colicinogenic factor appears with similar proportions among recombinants integrating proximal or distal markers from any of the different Hfr strains employed.

These data indicate that the colicinogenic factors studied are not linked to *thr—leu*, *his*, *try*, or *arg* markers, which are localized at different sites along the linkage group of *E. coli* K12 (text-fig. 1). Moreover, with *col E*$_2$, *col V*, and *col B*, the transfer of colicinogeny seems to be proportional to the duration of conjugation, since recombinants for distal markers present a higher percentage of *col*+ than those for proximal ones.

In reciprocal crosses, involving noncolicinogenic donors and colicinogenic recipients, transfer of the noncolicinogenic character of the donor to recombinants never occurred. Nor was any death of zygotes related to a presumptive transfer of a *col*— locus observed in these same crosses performed in the proportion of 20 donors to 1 recipient cell. Therefore, there are no reasons to consider the nonappearance of the *col*— character among recombinants as the consequence of some type of lethal zygosis masking its transfer.

Therefore, analysis of crosses between colicinogenic donor and noncolicinogenic recipients indicates that: 1) Each colicinogenic factor is transferred with similar frequencies whatever the type of donor bacteria involved; 2) they present similar times of entry from these different donor bacteria; and 3) they show no linkage with chromosomal markers.



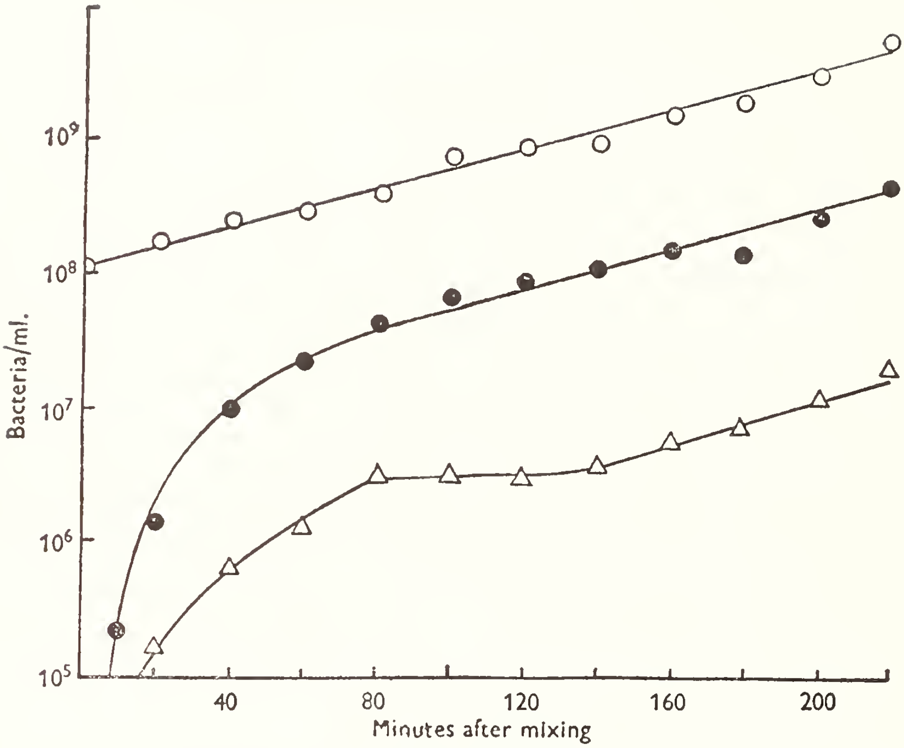
Text-figure 1.—Linkage map of *Escherichia coli* K12 (Jacob & Wollman, 1961) with indication of the origin and direction of the Hfr employed. For abbreviations, *see* footnote table 1.

It may be concluded from these observations that colicinogenic factors V, E_1, B, E_2, and I are not localized on the chromosome. If a chromosomal locus existed, quite different efficiencies of transfer and different times of entry would be expected among Hfr strains differing in origin and polarity (as shown with the Hfr strains in text-fig. 1) and also between Hfr strains, which transfer the chromosome at high frequency, and F+ strains that transfer it at low frequency.

That the *col*− character of the donor is not transferred to recipients can also be explained on the basis of the extrachromosomal state of colicinogenic factors. Evidence from kinetic experiments with *col* E_1, *col V*, and *col B* factors also strengthen this conclusion. As seen in text-figure 2, where kinetics of transfer of *col* E_1 are presented (*4*), *col*+ str^r cells (combining the *col*+ character of the donor and the str^r character of the recipient) do not reach the plateau characteristics of chromosomal recombinants between 80 and 120 minutes. This indicates that, in contrast to chromosomal markers, the *col* factor once transferred to the zygote can multiply immediately, without integration, and at a rate that assures its passage to all daughter cells.

2. Interactions Between Colicinogenic Factors and Fertility Factors

During our studies with colicinogenic factors, certain of their interactions with fertility factors became evident. The presence of colicino-



TEXT-FIGURE 2.—The kinetics of transfer of *col* E_1 and *thr*+ *leu*+ in Hfr H *col* E_1+ × PA 309 *col* E_1 cross, performed in the proportion of 1 donor to 20 recipient cells. Samples were taken at different times, treated in a Waring blendor, diluted, and and plated on selective media. A sample of the mating mixture was diluted 1/10 in fresh nutrient broth every 100 minutes to maintain it in the logarithmic phase of growth. △, *thr*+*leu*+*str*^{r} recombinants; ●, *col*+*str*^{r} bacteria; ○, *str*^{r} (F) bacteria.

genic E_1, E_2, or I factors did not interfere with chromosomal transfer or integration, as shown by the fact that similar frequencies of recombination were observed in crosses involving either colicinogenic or noncolicinogenic donors (table 4). This was not the case with colicinogenic factors B or V, whose presence in donor cells drastically reduced the frequency of recombinant formation (*5–7*). No variation in fertility was observed when only the recipients were colicinogenic for B or V. As shown in table 5, strain F+ 112 (*col V–col I*)+ presents a marked decrease in chromosomal fertility as compared with the original frequency. Since we found that all our *col V*+ strains synthesized besides colicin V another colicin, identified as colicin I, we will refer to these *col V*+ strains as (*col V–col I*)+.

An interference with fertility was also manifested by *col V–col I* factors with different Hfr strains. In these instances, a reduction in the frequency of recombinants by a factor of 10^3 to 10^5 was observed, though the gradient of transmission of markers of the original Hfr strain was maintained.

TABLE 4.—Comparison of fertility in crosses involving colicinogenic factors, with control crosses

Crosses	Frequency of recombinants for an early Hfr marker (%)
Hfr P4X *col* E_2+ × F- *col* E_2−	25
Hfr P4X *col* E_2− × F- *col* E_2−	21 (control)
Hfr P10 *col I*+ × F- *col I*−	18
Hfr P10 *col I*− × F- *col I*−	24 (control)
Hfr AT IIIA *col* E_1+ × F- *col* E_1−	25
Hfr AT IIIA *col* E_1− × F- *col* E_1−	23 (control)
Hfr H *col V*+ × F- *col V*−	40
Hfr H *col V*− × F- *col V*−	35 (control)

Because of this interference with fertility and in order to follow the fate of the F factor upon entrance of *col V–col I*, an F–*lac*+ strain was used as recipient. In F–*lac*+ strains, the F factor can be easily followed by detection of the *lac*+ character, since both the *lac*+ and the F factor are closely linked. As can be seen in text-figure 3, the acquisition by this F–*lac* strain of the V–I colicinogenic character was accompanied by loss of the F–*lac* factor as evidenced by the fact that increase of (*col V–col I*)+ str^r cells equals the increase in *lac*–str^r cells. This result suggests that the entrance of *col V–col I* factor inhibits in some way the multiplication of the F–*lac* factor, and thus leads to its loss.

Though most bacteria in an F–*lac*+ population receiving the *col V–col I* factor became *lac*−, we isolated some in which *lac*+ and colicinogenic factor V–I coexisted. To determine the state of the *lac* factor in these strains, they were used as donors in matings. We found that these (*col V–col I*)+ *lac*+ bacteria, like other (*col V–col I*)+ donors, presented a marked decrease in chromosomal fertility though they transferred (*col V–col I*) at high frequency. The *lac*+ character, however, which was transferred at high frequency from the original F–*lac* donor strain, was not transferred at all by these strains (table 5). Similar results were obtained when F–*gal* strains were used instead of F–*lac* strains (table 5).

These results, together with the fact that no segregation of the *lac*+ character was observed, suggest that in these *lac*+ (*col V–col I*)+ strains, *lac*+ is integrated in the chromosome.

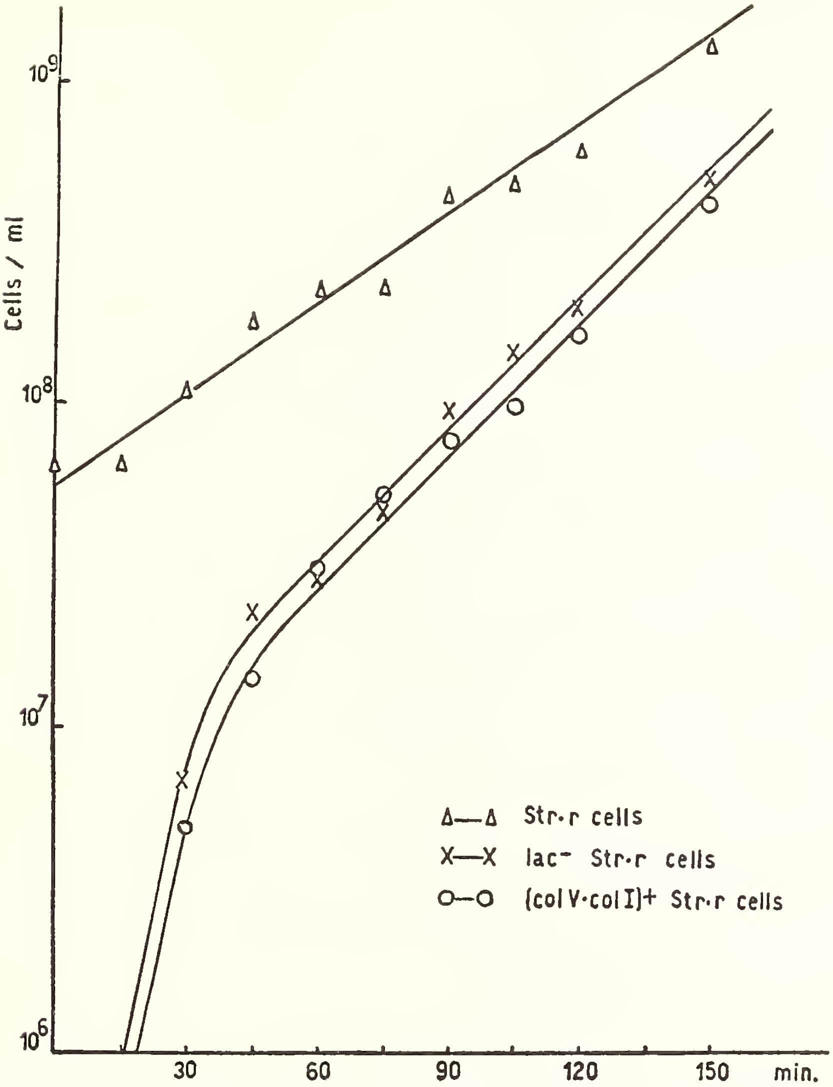
Interestingly enough, a few of these *col V–col I*+ *lac*+ were found to segregate *lac*−. Apparently then, *col V–col I* and F–*lac* can coexist in an autonomous state, though only in a small proportion of cells. But, as mating behavior indicates, even in these strains, F–*lac* is no longer transferred nor promotes the transfer of the chromosome.

On the other hand, we made several observations which suggest that *col V–col I* factor possesses some properties characteristic of fertility factors.

TABLE 5.—Transfer of *col V–col I* factors among *Escherichia coli* K12 strains*

Donors	Sexual type	*lac*+ or *gal*+	*arg*+	*try*+	*his*+	*thr*+ *leu*+	(*col V–col I*)+
112	F+		4×10^{-3}	1×10^{-2}		3×10^{-3}	—
112 (*col V–col I*)+			0	3×10^{-4}		2×10^{-4}	1500
200 PS F-*lac*+	F′	190				1	—
200 PS F-*lac*+ (*col V–col I*)+						0	500
W 3101 F-*gal*+	F′	120				1	—
W 3101 F-*gal*+ (*col V–col I*)+						2×10^{-5}	1000
C 600	F–		0	0	0		—
C 600 (*col V–col I*)+			3×10^{-4}	8×10^{-4}	2×10^{-4}		1000
W 3876	F–♀3		0	0	0		—
W 3876 (*col V–col I*)+			3×10^{-2}	1×10^{-2}	1×10^{-2}		200

* The donor parent was contraselected with streptomycin in crosses involving strains 112, 200 PS, W 3101, and C 600 and with valine with strain W 3876.



TEXT-FIGURE 3.—Kinetics of transfer of (*col V–col I*) factor to an F–*lac*+ strain.

Thus, 1) all F— cells acquiring *col V–col I* factor became sensitive to phage *MS2*, which is known to plate only on donor cells; 2) *col V–col I* factor was transferred from an F— strain to another with about the same efficiency as from F+ or Hfr strains; 3) the presence of *col V–col I* factor in an F— cell also appeared to confer chromosomal fertility, which though low was not evidenced in the control cross *col*— × *col*— (table 5); and 4) a higher chromosomal fertility was observed when the (*col V–col I*)+ F— strain used as donor harbored a chromosomal sex factor attachment locus, as shown with Richter's F— strain (table 5).

These results show that *col V–col I* factor interferes with chromosomal fertility in donor cells, though it is being transferred at high frequency.

Our results indicate that the presence of *col V–col I* factor in the cell confers on it the ability to conjugate, thus enabling its efficient passage to other F— cells, and, with very low efficiency, the passage of the chromosome as well.

Two different hypotheses accounting for the observed interactions with F factors can be formulated: 1) that *col* factors *interfere with the expression* of the F factor in donor cells. Thus, epistasis on the F factor might be complete or at least might affect the functions related to the transfer of the chromosome; or 2) that *they interfere with the presence* of the F factor in donor cells.

Results with F–*lac* and F–*gal* strains made (*col V–col I*)+ suggest that the exclusion between both types of factors occurs. According to the replicon model proposed by Jacob *et al.* (*8*), these interactions would reflect the competition for attachment to a few common sites for replication.

As noted before, analysis of the behavior of colicinogenic factors in mating led us to consider them as plasmids. Nevertheless, the possibility that some colicinogenic factors might become fixed on the chromosome under certain conditions cannot, at present, be excluded. Even though no evidence bearing on a direct chromosomal attachment of colicinogenic factors has been found, one may speculate that it might be more likely for colicinogenic factors like V–I and B that resemble F particles whose chromosomal location is well established. In this connection Fredericq (*9*) has reported the isolation of an Hfr strain carrying *col B* fixed on the distal end of the chromosome as a result of a close association with the F factor.

The behavior of colicinogenic factor V–I is strikingly similar to that of multiple drug resistance factors, intensively studied by Japanese workers (*10*). These factors, like colicinogenic factor V–I, are transferred with equal frequency from F+, Hfr, or F— cells; they confer capacity of mating on F— cells and interfere with the fertility of donor cells, but in contrast to colicinogenic factor V–I, they do not confer sensitivity to phage *MS2*.

Although colicinogenic factor V–I and multiple drug resistance factors are quite different cellular particles, these aspects of their behavior point not only to a similarity between them, but also with F′ factors, which suggests that they might all be related in their origin.

RESUMEN

Se ha estudiado el comportamiento genético de los factores colicinogénicos E_1, E_2, I, V y B en cepas de *Escherichia coli* K12.

Del análisis de cruzamientos que utilizan cepas Hfr y F+ como donantes es sededuce que:

1) Cada factor colicinogénico es transferido con frecuencia similar cualquier sea el tipo de la bacteria donante involucrada en el cruzamiento.

2) No hay ligamiento con marcadors cromosómicos.

3) El carácter no-colicinogénico del donante no se transmite nunca a los recombinantes y

4) Zigotas que han recibido el factor colicinogénico lo trasmiten a las células hijas.

Estos resultados llevan a la conclusión que los factores colicinogénicos están en estado extracromosómico en *E. coli* K12.

Por otra parte, los factores col V–col I poseen algunas propiedades características de los factores de fertilidad desde que células F— que adquieren los factores col V–col I se tornan sensitivas al fago MS2 y transfieren el carácter col V–col I con la misma eficiencia que cepas F^+ o Hfr.

Una interferencia con la fertilidad es manifiestada también por los factores (col V–col I) con diferentes cepas Hfr y F^+, desde que sus presencias en estas cepas reducen drásticamente la frecuencia de formación de recombinantes.

Resultados obtenidos con una cepa F—lac^+, hecha (col V–col I)$^+$ indicar que la adquisición por esta cepa de factores colicinogénicos está acompañada por pérdida del factor F— *lac* sugiriendo que la entrada del factor col V–col I inhibe de alguna manera la multiplicación del factor F—*lac*.

Estos hechos son discutidos en conexión con una posible relación entre colicinógenos, resistencia a drogas múltiples y factores de fertilidad.

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DISCUSIÓN

Wollman (Institut Pasteur, Paris): Quisiera preguntarle dos cosas, una con respecto a la disminución de la frecuencia de recombinación, la otra con respecto a la polaridad. Tiene Ud. alguna idea de cuál es el factor que actúa en cada uno de esos efectos, porque se conoce que el factor col I confiere polaridad, podría ser que el factor col V determine la supresión de la polaridad sexual en estas cepas.

de Zwaig: Hasta ahora no hemos podido separar el factor colicinógeno V del factor colicinógeno I, por lo tanto no sabemos cual es el responsable de los efectos de interacción con la fertilidad y de dar fertilidad. Hay antecedentes por trabajos de otros autores de que el factor colicinógeno I actúa como un factor de fertilidad, pero dado que las cepas de las cuales provieneos estos factores no son las mismas, no podemos asegurar que se trate en este caso del mismo factor I. Se podría especular también sobre la posibilidad de que ninguno de los dos, ni col V ni col I sean por sí solos los factores responsables de la fertilidad, sino que estos dos factores estén unidos a un tercer factor que sea el que de sea capacidad de transferencia. De ser así, este caso sería muy similar al de los factores de resistencia multiple a las drogas, con los cuales se logró separar el factor responsable de la transmisibilidad de los que confieren la resistencia a las drogas.

Chromosome Structure and Replication

The Bacteriophage Chromosome [1, 2]

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SUMMARY

This paper reviews briefly some of the recent discoveries concerning the physical and genetic structure of bacteriophage chromosomes. Evidence for the occurrence of a ring form at some stage of the life cycle has been found for the phages ϕX, lambda, and indirectly, T4. Physiological studies of conditional lethal mutations have revealed a high degree of order with respect to function of the gene complements of phages T4 and lambda. Some implications of these findings are discussed.—Nat Cancer Inst Monogr 18: 67–77, 1965.

IN THE LAST few years, there has been a rapid increase in our knowledge of the organization of viral genomes. Physical studies indicate that viral nucleic acids exhibit surprising structural complexity and individuality, and genetic analysis has revealed a high degree of order with respect to function in the arrangement of the gene complement. This paper surveys briefly some of these salient features of bacteriophage "chromosomes."

PHYSICAL STRUCTURE

The bacteriophages (and plant and animal viruses) that have been carefully examined contain but one nucleic acid molecule which can vary in size by almost two orders of magnitude. The deoxyribonucleic acid (DNA) of phage ϕX contains 5,500 nucleotides (*1*), while that of T4 has about 200,000 nucleotide pairs (*2*). This great range in size reflects great differences in gene number, since one can compute that the small viruses, such as ϕX, can have little more than 6 genes, while T4 must have 100 or more. This agrees with the finding of mutations in only 3 or 4 genes in the small phage f2 (*3*), whereas mutations in more than 60 genes have been found in phage T4 (*4, 5*).

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

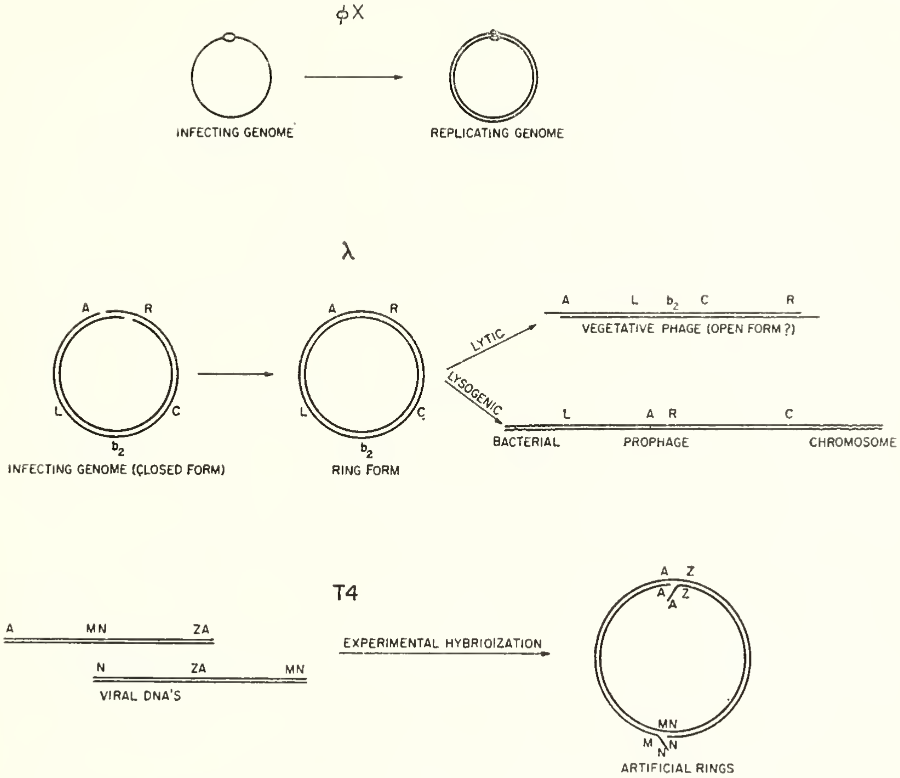
[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

Perhaps the most surprising discovery in recent years is that, in one way or another, "circularity" enters into considerations of genome structure in phage. A ring structure for the single-stranded DNA of phage ϕX was inferred from physicochemical studies (*6*) and has now been verified by electron microscopic studies (*7*). Upon infection of host cells, the single-stranded ring is converted into a double-stranded ring which has also been identified as such with the aid of the electron microscope (*8*). Studies on enzymatic digestion of linear ϕX DNA molecules reveal a unique "discontinuity" in the ring which blocks the action of exonucleases (*6*). This discontinuity may well be some kind of "linker," chemically distinct from the rest of the polynucleotide structure.

Although the DNA molecule isolated from phage lambda is linear, its ends are "sticky" and thus it can form ringlike configurations (closed form) under appropriate conditions (*9*). Recent evidence indicates that the sticky ends are complementary single strands which in low salt can anneal (*10*). When cells are infected by phage lambda, DNA molecules that appear to be covalently bonded rings are formed (*11*). The fact that the linkage structures of the vegetative phage and the prophage, which is integrated into the bacterial chromosome, are both linear but circular permutations of one another also suggests the presence of a circular intermediate in the life cycle of lambda (*12*).

One might imagine that the nucleic acid of viruses is entirely concerned with the encoded information required for their growth. Yet, at least for lambda, deletion mutants lacking large segments of DNA, which are nevertheless viable, have been found (*13*). The b2 mutant of lambda, for example, contains 15 percent less DNA than the wild type, as determined by DNA measurements (*14*) and "cytogenetic" studies [length measurements from electron micrographs of mutant and wild-type DNA (*10*)]. The function of these dispensable genome segments remains to be elucidated. The $b2^+$ region of lambda may represent a pairing segment involved in the association of the phage genome with the bacterial chromosome, since the b2 mutant does not become integrated into the bacterial chromosome although it can establish an abortive lysogeny (*15*).

Although the genetic linkage map of phage T4 is circular (*16*), the DNA molecule of the mature phage is linear (*2*). This apparent discrepancy has been explained by the assumption that the DNA molecules isolated from phage particles are circular permutations of the genetic structure (*16*). Various physical studies of T4 DNA support this proposal (*17*). The most convincing is perhaps the formation of ring structures in reannealed preparations of single strands (*18*). Genetic evidence suggests that each molecule contains slightly more than a full complement of genes resulting in structures with a terminal redundancy (*e.g.*, abc . . . yza; mno . . . klm, etc.) (*19*). The fact that the viral DNA molecules are circular permutations of the genome suggests that perhaps in this phage, as well, circularization of the genome occurs at some stage of the growth cycle (text-fig. 1).



Text-figure 1.—*Top.* ϕX phage DNA, single-stranded with a "discontinuity," and the double-stranded "replicative form." *Middle.* Lambda DNA, a linear molecule with hydrogen-bonded complementary ends in the "closed form." Upon infection, the molecule probably becomes a closed ring that assumes two linear forms upon integration into the bacterial chromosome or vegetative multiplication. *Bottom.* T4 DNA molecules as circular permutations of the genome with a terminal redundancy. If the strands are separated and reannealed, artificial ring molecules are formed.

This brief description of some of the physical properties of the nucleic acid component of three of the more intensively studied bacteriophages indicates that phage chromosomes are not simply nucleotide polymers but are organelles endowed with special structural features: linkers, sticky ends, terminal redundancies, and dispensable segments. Although circularity must play a significant role in the replication cycle of viral nucleic acids, it remains to be revealed. The circularity of the genome of the bacterium *Escherichia coli* (*20*) suggests the possibility that circularity may occur, at least at some level of organization, in the structure and replication of chromosomes in higher organisms (*21*). Disconcerting is the variety of form and of replication pattern assumed by the few phages studied. Hopefully, further work on a variety of phages will reveal the underlying significance of this individuality.

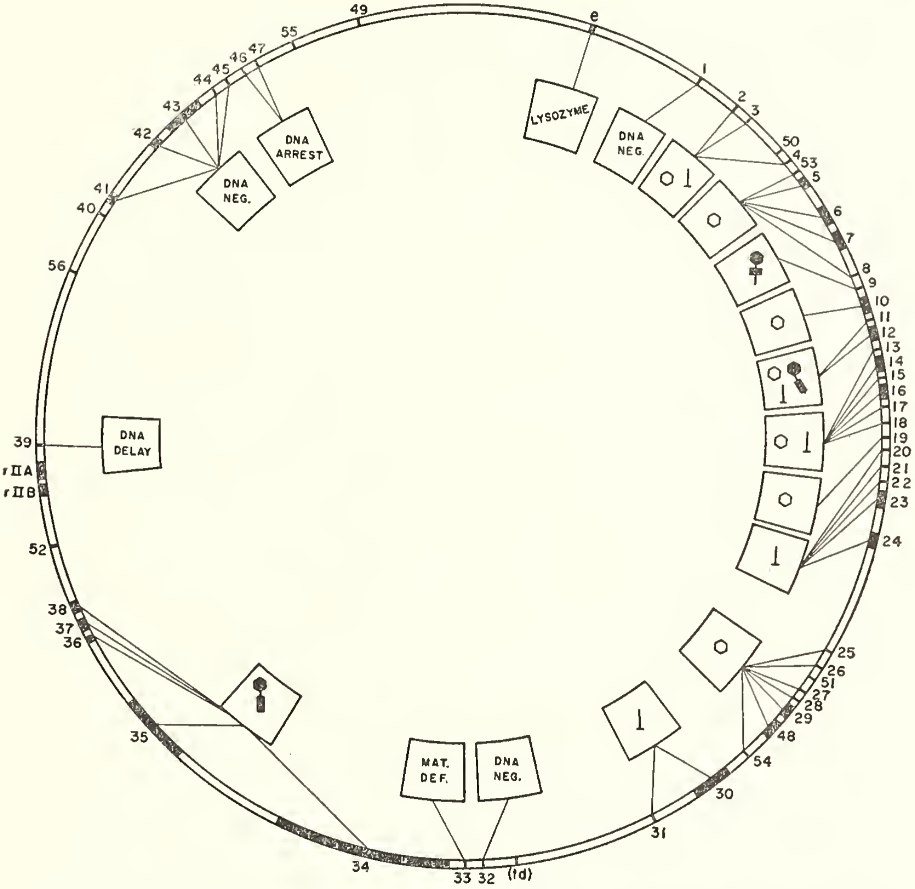
FUNCTIONAL ORGANIZATION

For many years analysis of the functional properties of viral genomes was hampered by a lack of suitable mutations. Recently the development of conditional lethal systems has permitted a detailed analysis of the genetic structure of phage from a physiological point of view. The pioneering studies of Jacob *et al.* (*22*) on defective lambda prophage mutants suggested that the genes of the virus are not randomly arranged, but organized into clusters reflecting common functions. More recently, studies of temperature-sensitive (*23*) and amber (*4*) mutants of phage T4 and of suppressor-sensitive mutants of lambda (*24*) have extended these initial observations.

Physiological studies of the growth of T4 show that there are at least two stages to the growth cycle—an "early" stage characterized by the synthesis of proteins largely concerned with DNA synthesis, and a "late" stage during which the protein components of the phage are synthesized. It is not known if within these two periods there is a sequential appearance of different protein species. To date, over 60 genes, probably half the total complement, have been identified and their functions partially established (*25*). There is no obvious correspondence between the gene arrangement and the sequential activities of the genes. Genes appearing to act during early stages of growth (genes 39–49, 1, 32–33) are located in three regions separated by genes which act during the later stages of the growth cycle. Although there appears to be no direct relationship of gene order to time of function, it is quite apparent that the genes are not randomly arranged. They occur rather in clusters of common phenotype (text-fig. 2).

These clusters are reminiscent of those found for genes controlling the enzymes of biosynthetic pathways in bacteria and are explained by the operon theory (*26*). In the case of phage, however, many of the clusters involve genes concerned with the morphogenesis of the virus particle. At present, there is no evidence of any functional interaction between mutations in different genes but in the same cluster. The autonomy of functional behavior of the mutants (*4*) and the refractoriness of almost all of the gene products to biochemical analysis make it difficult to determine the significance of the clustering.

One morphological component for which there is a ready assay is the tail fiber. Serological studies with mutants of the group of genes which control tail fiber synthesis reveal the existence of at least three distinct tail fiber antigens (*27*). Table 1 indicates the role various genes defective in tail fiber synthesis play in the formation of these three antigens. Unfortunately, there is no assurance that the antigens are primary gene products. The fact that several of these antigens require the activity of more than one gene for their appearance suggests that they are not simple assays of primary gene products. Interestingly, phages defective in gene 57 are lacking in the production of all three antigens although phage particles are made. This gene, located near gene 1 (not shown in text-fig. 2), is



Text-figure 2.—The map of T4. The phenotypes of mutants defective in the various genes are indicated in the boxes. DNA NEG.: no DNA synthesis; DNA ARREST: DNA synthesis starts but is arrested after a short time; DNA DELAY: DNA synthesis starts only after an initial delay; MAT. DEF.: DNA synthesis is normal but there is a general suppression of late functions; LYSOZYME: no active endolysin produced. Other symbols indicate the types of phage components produced in normal abundance, *i.e.*, head membranes, tails, or particles lacking fibers.

distantly linked to the other tail fiber genes. An appealing explanation of this finding is that gene 57 has a regulatory function, controlling the expression of the genes of the tail fiber cluster; other solitary genes may act similarly (*cf.* genes 1, 10, and 20). If this gene is in fact a regulatory gene, it differs in nature from those found for the operons of bacteria since this gene in its inactive rather than active form results in the lack of activity of the gene cluster it controls. Clearly, these remarks are speculative and more evidence must be obtained to determine whether this gene plays a regulatory role.

Another morphological component that can be studied both genetically and biochemically is the head. The head membrane is composed of one main protein subunit. This protein has been shown to be under the control of gene 23 (*28*). Mutations in genes on either side of gene 23 do not effect the synthesis of head protein, only its assembly into normal head membrane (*29*). The role of other genes (20, 21, 22, 24, 30, and 31) in head morphogenesis is still obscure.

Twenty-one genes have now been identified in phage lambda and at least partially characterized as to function (*24*). As indicated in text-figure 3, the map of lambda can be drawn as circular with two derivative linear forms—the vegetative and prophage. If the map is drawn in the circular or the prophage form, a striking correspondence between the relative locations of genes and the sequence of their activities in the growth cycle of the phage may be seen.

TABLE 1.—Production of tail fiber antigens under restrictive conditions

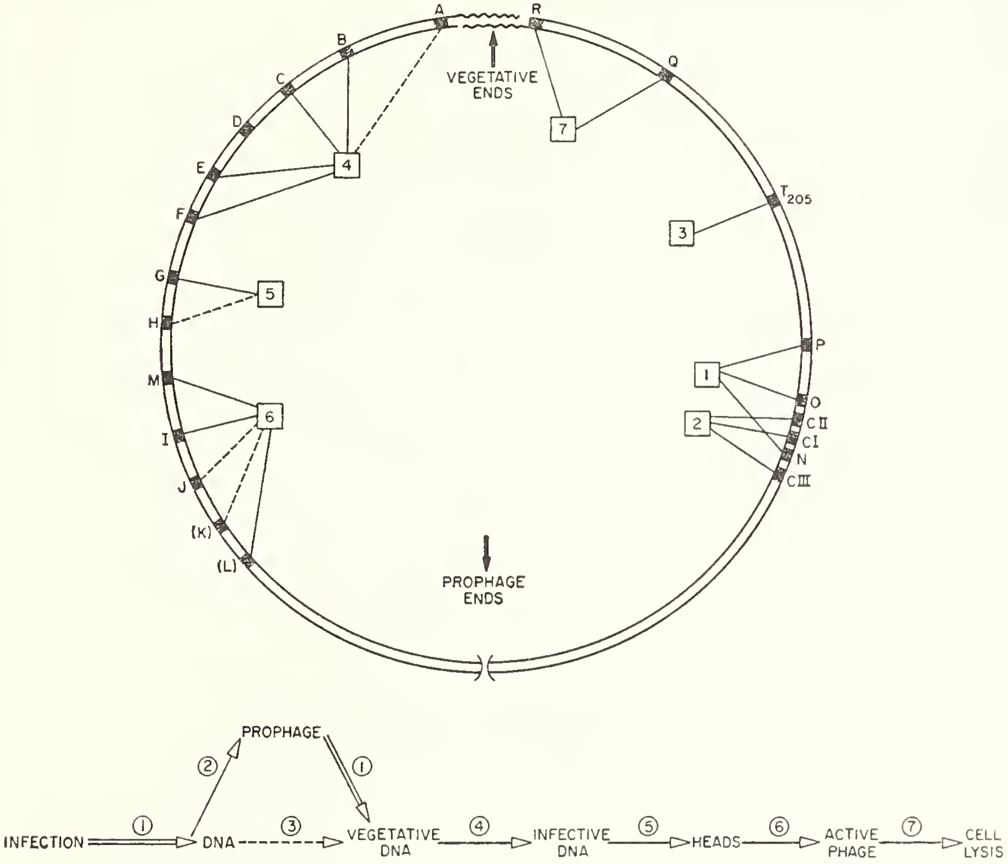
Gene defective*	Antigens		
	A	B	C
None (+)	+	+	+
34	0	+	+
35	+	+	+
36	+	0	+
37	+	0	0
38	+	0	0
57	0	0	0

*Results refer to production of antigens by amber mutants in the restrictive host *Escherichia coli* strain B.

In one region of the map are the genes involved in early steps in development. Genes *CII*, *CI*, and *CIII* are concerned with the establishment of lysogeny, though not essential for lytic growth. Genes *N*, *O*, and *P* are necessary in active form for replication of lambda genomes within the infected cell (*30*). [Gene *N* appears to control the synthesis of a lambda-induced exonuclease (*31*).] Genes *Q* and *R* are involved in the process of cell lysis, *R* in a direct way by determining the structure of the phage endolysin (*24, 30*). Although cell lysis is a terminal step in development, the synthesis of the endolysin, at least, is initiated some time earlier.

Although DNA extracted from lambda phage particles is infective, vegetative lambda DNA is not infective (*32*). Thus a transformation of the noninfectious vegetative DNA to its infectious form must occur as a step in maturation. This transformation is possibly correlated with the formation of head membranes in the cell and the condensation of the viral DNA. The results shown in text-figure 3 suggest that mutations in genes *A* through *F* produce lesions in the maturation process prior to the appearance of infective DNA (*33*). Genes *M* through *L* appear to be involved in later stages in maturation, presumably tail formation, since head membranes and infective DNA are formed by these mutants (*33*). The fact that the host range mutation maps to this region of the genome supports this notion.

Although it should be stressed that some of the observations reported in the table are unpublished, the available evidence suggests that the sequence of genes on the lambda map is correlated with the order in



Text-figure 3.—A map of lambda. The ends in the prophage and vegetative phage are indicated. The numbers refer to blocks in the developmental pathway as indicated in the text, *e.g.*, mutants of type 1 are blocked in all processes; type 2 in lysogenization only; type 3 mutants can lysogenize and grow vegetatively after induction but not after infection—these have recently been isolated by Réné Thomas; mutants of type 4–6 do cause cell lysis but are blocked at various stages of maturation.

which these genes function in the phage growth cycle. It should be noted, however, that although the phenotypic sequence observed is one reflecting the sequence of steps in the developmental pathway, the sequence in which the gene proteins are synthesized does not necessarily follow the same time course. This pattern was not observed for phage T4. Since this orderly pattern cannot be readily perceived for the vegetative map of lambda, it is possible that the activities of the genes may be determined at a stage when the genome is in a circular form.

CONCLUDING REMARKS

For phages T4 and lambda, roughly half of the genes have been "tagged" with mutations rendering them amenable to physiological analysis.

Consequently, we are beginning to perceive the over-all design of these genomes and the complexities of the many functions they perform. Our knowledge of the genomes of other organisms is much more meager, since it is, in most cases, based almost entirely upon studies of genes determining nutritional properties. Even in as simple an organism as a bacterium it is likely that the latter kinds of genes constitute less than 10 percent of the gene complement. Most genes remain unidentified and their functions unknown.

The conditional lethal systems developed for phage, particularly the temperature-sensitive system, are applicable to other organisms. Although even bacteria are roughly an order of magnitude more complex than phage, sampling techniques might well suffice to give some insight into the organization and functional properties of that major portion of the genome which has not yet been explored. Physiological studies of non-nutritional mutants of simple organisms might aid in revealing the types of functions expected for genes in organisms slightly higher in the phylogenetic scale. In this way one might hope to apprize the organization and functional complexity of the genomes of higher organisms as of now only dimly perceived.

RESUMEN

Este estudio analiza brevemente algunos de los recientes descubrimientos con relación a la estructura física y genética de los cromosomas del bacteriófago. Se ha puesto en evidencia la presencia de cromosomas en forma de anillo en alguna de las etapas del ciclo de vida de los fagos ϕX, lambda y T4. Estudios fisiológicos de las mutaciones letales condicionales han revelado un alto grado de orden con respecto a la función de los complementos génicos de los fagos T4 y lambda. Son discutidas algunas implicaciones de dichos hallazgos.

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DISCUSSION

Bozzini: Taking into account the circularity of the chromosome you have shown, do you think that physical recombination as far as it is known for T4 and lambda is similar or is it quite different from one phage to the other?

Edgar: I would hope that there are common features to recombination mechanisms in all organisms. Nevertheless, there are certainly striking differences in recombination mechanisms. In certain phages, for instance, the frequency of heterozygotes is markedly different. Hopefully, when we know more about recombination, we will be able to account for the differences and be aware of the common features that exist.

Pavan: I was very surprised to hear of the b2 mutation which has a deficiency of 15 percent of the phage's DNA. It is hard to understand how a phage still can live after losing 15 percent of its genetic information. From what I could see of your map, there are several mutations causing a DNA negative situation. I would like to have some more information about this. Should we understand that the phage lives in a much better environment than the higher organism, being inside the cell? In higher organisms one can get very small deficiencies that are lethal in the homozygous condition. The only exceptions are in some heterochromatic regions. Could we infer the presence of dispensable genes in a phage or something like heterochromatin in their chromosomes?

Edgar: I certainly think it is surprising that such a large fraction of the DNA can be dispensed with. In the case of b2 mutation, the phage can establish lysogeny but it cannot integrate into the chromosome and so a sort of abortive lysogeny results. As a consequence, this is possibly some kind of pairing segment and when it is deleted all the phage functions are still performed by the lambda except this critical one of being able to integrate. However, there are many other mutants known both in lambda and in T5 and possibly T7 which could be DNA deletions. Apparently there is a fair amount of the DNA of various phages that can be dispensed with without affecting viability. Whether this is a loss of genes which are, let us say, essential in the "wild environment" of the phage but not in the laboratory or whether these are segments of DNA that have functions different from normal gene functions, I cannot say. I hope that the study of these deletion mutations will be continued.

Reissig: Can you make an estimate of how long it takes to read the genome in relation to the duration of the life cycle?

Edgar: Only in the case of T4 is enough known to make even a guess, and the problem is complicated by the fact that there is not a simple sequential expression of the genome. There are two stages of expression, the early stage and the late stage, and some of the genes are active during early stages and some during late stages. It is not resolved yet whether this regulation phenomenon is at the messenger level or at some other level. RNA messenger is being synthesized throughout the life cycle but it may well be that different genes are active at different times in this process.

Giles: I wish to ask about the clustering of genes controlling similar phenotypes. You mentioned that there is apparently little evidence that these are organized as operons. Is this a result of a lack of sufficient information or is the method of complementation analysis such that you would not pick up such functionally related higher groupings of genes?

Edgar: Our studies have been only of conditional lethal mutants—temperature-sensitive mutations and amber mutations. Extensive complementation studies indicate that various genes behave as classical genes, the temperature mutants showing intra-genic complementation and the amber mutants none; mutations in different genes but within the same cluster appeared to show complete complementation. Furthermore, there are several cases where we can look at the amount of protein made by a particular gene when genes on either side are mutant, and so far there have been no obvious differences found. Thus far, no evidence for any kind of polarity effect has been seen. However, this may well be due to the fact that we are only looking at a selected class of mutants. Perhaps most or all of these are nonpolar mutants.

Schultz: I should like to bring up the question of the appropriateness of the use of the term "chromosome" in these contexts. Obviously the studies of the phage and other viruses supply us with some of the principles on which we can base an analysis of the grosser phenomena in the chromosomes of mitotic types. But I wonder whether we serve any purpose by using the same names for structures that differ so much in order of magnitude. For you, T4 is a giant but T4 has only, when one calculates, about 5 to 10 times the number of nucleotide pairs that the finest of the salivary gland bands has when reduced to ultimate haploid value in the *Drosophila* genome. These giants, thousands of them, are put together in the complex structure called a chromosome. How such operations are derived from the more closely analyzed viral principles we have to discover; to call the two things by the same name may be a real disservice to the analysis. I do not have a better name at this point but I do believe we have to keep our terminology clear.

Fine Structure of Lampbrush Chromosomes [1, 2]

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SUMMARY

A surface-film method and a microcentrifugation method for preparing lampbrush chromosomes for electron microscopy are described. Results obtained with *Triturus viridescens* chromosomes show that the minimum diameter of deoxyribonuclease-sensitive lateral loop axes (1 chromatid wide) is 20 to 30 A, whereas deoxyribonuclease-sensitive interchromomeric strands in the main chromosome axes (2 chromatids wide) measure 30 to 50 A. These diameters are similar to those obtained by other methods for single double-helix deoxyribonucleic acid or deoxyribonucleoprotein molecules and indicate that the chromatids of these chromosomes consist in width of single deoxyribonucleic acid or deoxyribonucleoprotein molecules. Three types of ribonucleoprotein matrix were observed on lateral loops. The only morphological change occurring in individual matrix units from thin to thick insertion ends of loops is an increase in length, suggesting that synthesis of ribonucleic acid molecules starts at the thin end of loops and continues by a zipper-like action around the entire length of a stationary loop axis. The longest matrix units are longer than would be predicted from lengths of ribonucleic acid molecules estimated by other methods, suggesting that the ribonucleoprotein fibrils on these chromosomes may be subdivided into smaller units before functioning in protein synthesis. The longest matrix units observed are much shorter than loop axes, suggesting either that the entire length of the deoxyribonucleic acid in loop axes may not be used for transcription or that the length of loop axes does not consist entirely of deoxyribonucleic acid.—Nat Cancer Inst Monogr 18: 79–99, 1965.

THE LAMPBRUSH chromosomes of amphibian oocytes can be readily isolated in a condition which closely resembles their appearance in the living cell. These meiotic chromosomes are relatively uncoiled and in a highly extended state when compared with chromosomes in other cell types. A number of both light and electron microscope investigations of lampbrush chromosomes [*see* (*1*, *2*) for reviews] have given rise to the concept of structure depicted in text-figure 1 and allow the following brief description. Lampbrush chromosomes are long (up to 1 mm in length)

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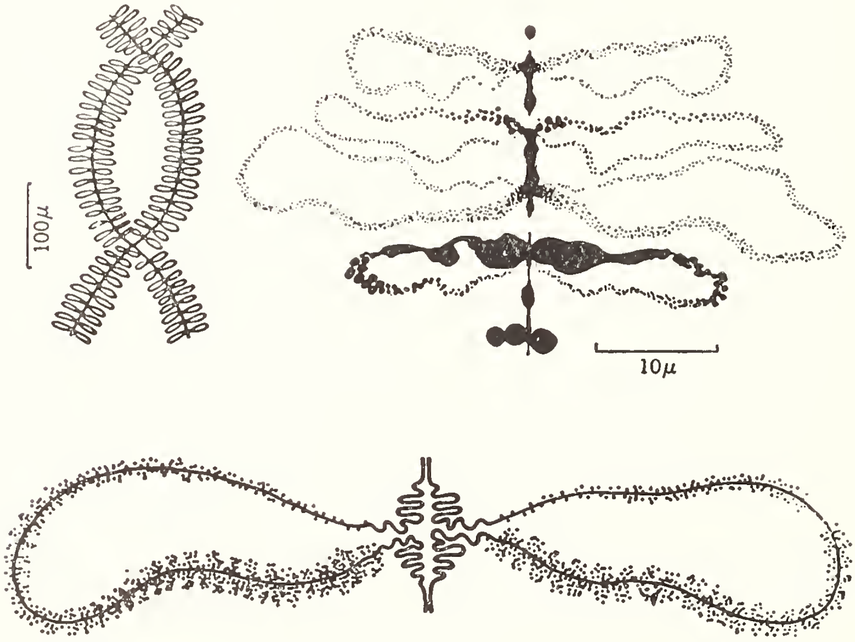
[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

diplotene bivalents joined by 1 to several chiasmata. The main axis of each homologue, which contains 2 chromatids, consists of a series of closely spaced chromomeres connected by a thin fiber. The chromatids in the main axis separate in the chromomere regions to form the axes of pairs of lateral loops which are coated with a ribonucleoprotein (RNP) matrix. Paired loops are similar in morphology, but the loops may vary in morphology from one pair to the next. Each loop is asymmetric, having a thick and a thin end at the point of insertion into the main chromosome axis. The loop axes and interchromomeric threads can be broken by deoxyribonuclease (DNase) but not by proteases or ribonuclease (RNase). The loop matrix material can be removed with proteases or RNase but is not deformed by DNase except through the ultimate destruction of the axis to which this material is attached.



TEXT-FIGURE 1.—*Upper left:* diagrammatic sketch of a pair of lampbrush chromosomes joined by 2 chiasmata. *Upper right:* drawing of pairs of loops showing differences in morphology and length. *Bottom:* concept of continuity of chromatids in main chromosome axis with those forming axes of the lateral loops. [From Swanson (*3*), after Gall (*4*).]

Labeling with radioactive precursors shows that continual, rapid synthesis of both RNA and protein is occurring on these chromosomes. Thus, the lampbrush chromosome offers a unique opportunity for critically examining both basic chromosome structure and structural aspects of at least one chromosome function—the transcription of RNA.

Techniques for isolating lampbrush chromosomes for light microscopy have been described in detail (*5*). In general, however, these chromosomes have not been amenable to critical observations with the electron microscope *(6–8)*, either because of their compactness and high electron opacity following precipitation and drying, or because of coprecipitation of contaminating nucleoplasm onto specimen grids along with the chromosomes. This report describes innovations in preparing lampbrush chromosomes for electron microscopy and presents results obtained with these procedures. Oocytes from the common spotted newt, *Triturus viridescens*, were used for the study.

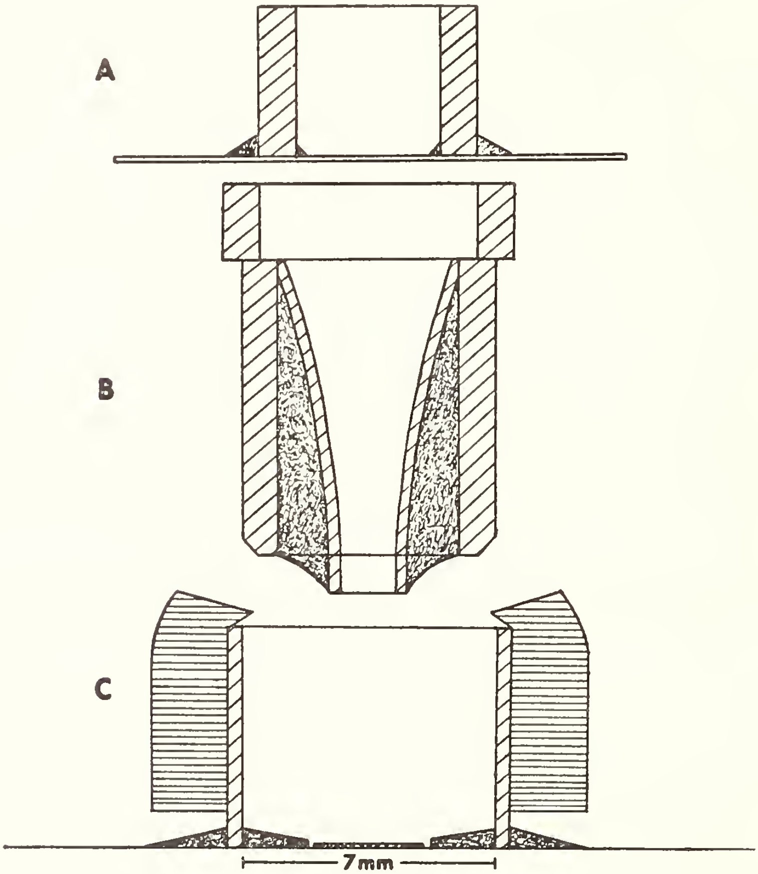
PREPARATION OF LAMPBRUSH CHROMOSOMES FOR ELECTRON MICROSCOPY

Surface-Film Method

With the aid of jewelers' forceps, the nucleus of a lampbrush-stage oocyte is isolated in saline and washed free of yolk with a braking mouth pipette. At this point, the nucleus can be treated with various agents or exposed to changes in *p*H or molarity, etc., which affect the structure of the chromosomes while still in the nucleus. After the nucleus has become somewhat swollen and turgid, it is pipetted into a small drop (2–5 mm diameter) of saline or 5 to 10 percent sucrose solution on a clean microscope slide. When sucrose is used, the nucleus usually will rise until the nuclear envelope attaches to the surface film; with saline, the drop is pulled down until the surface film contacts the nucleus. In both cases, surface tension forces burst the envelope and pull it, as well as a portion of the chromosomes and nucleoplasm, into the surface film. It is important to keep surfaces of all solutions free of oils or other substances which reduce surface tension, or no spreading occurs. After a nucleus has spread on the surface, the material in the surface film is picked up on a carbon-coated electron microscope grid by lightly touching the grid to the surface of the drop. The preparation then may be treated with enzymes and stained, etc., before drying and viewing in the electron microscope. Since soluble proteins and particulate materials from the nucleoplasm, as well as sheared-off chromosomal matrix material, are also retained on the grids, an objectionable background debris prevents optimal viewing in preparations obtained by this method. However, the method does allow observation of the deoxyribonucleoprotein components of loops because matrix material is removed from loop axes during the spreading process. Also, the surface-film action frequently uncoils or straightens individual units of the RNP matrix material.

Microcentrifugation Method

The centrifugation apparatus required for this technique consists of three components (text-fig. 2). The upper chamber (A) is made by attaching a short length of glass tubing to a coverslip with epoxy resin. The middle chamber (B) is constructed by gluing a funnel into another short length of glass tubing. The funnel can be made from the highly tapered section of a disposable Pasteur pipette. The wide mouth of the funnel should be flush with and exactly fill the glass tubing at one end, and the bottom end of the funnel should extend a little below the end of the glass tubing. A collar of glass tubing is glued on top of chamber B. The bottom chamber (C) is made by gluing to a microscope slide a short length of a rigid, thin-wall, plastic centrifuge tube which precisely accommodates the middle chamber. A short section of rubber tubing is slipped over the plastic section until only a small portion remains above the top of the plastic chamber. The tight rubber lip acts as a gasket and prevents liquid from being forced from the bottom chamber during centrifugation.



Text-figure 2.—Diagram of components of microcentrifugation tube used for preparation of lampbrush chromosomes for electron microscopy. *See* detailed explanation in text.

The components of the centrifugation apparatus are thoroughly cleaned, and a washed oocyte nucleus is placed in the medium of choice in the upper chamber. The chromosomes are manually isolated in the usual manner, and dispersal of the nuclear contents is observed with an inverted phase contrast microscope. Next, chamber C is filled with the centrifugation medium of choice and an electron microscope grid centered in the bottom of the chamber. Chamber B is inserted into Chamber C until the narrow end of the funnel is in contact with and centered on the grid. The level of the centrifugation medium is adjusted until the surface is slightly rounded above the top of the middle chamber.

After the chromosomes and nucleoplasm have sufficiently spread across the surface of the coverslip of chamber A, the surface of the medium in this chamber is adjusted until it is slightly rounded above the rim of the glass tubing. Chamber A is then inverted and quickly slipped into the top collar of chamber B, with care taken to prevent air bubbles from entering the apparatus. The slide holding the assembled components is placed into a flat cardboard holder mounted on the top of a multiple centrifuge tube carrier. The chromosomes then are centrifuged in a Size 1 Type SB International Centrifuge or equivalent for 3 to 4 minutes at a speed of 1000 to 2000 rpm. The two top chambers are then removed, and the grid with attached chromosomes is treated as desired before viewing in the electron microscope.

With this method, the chromosomes can be separated from essentially all of the background material inherent in the surface-film technique. In addition, the loop matrices can either be kept intact during the procedure or partially removed during or after centrifugation.

Following both methods, preparations were dehydrated in ethanol and air-dried from isopentane to minimize surface tension distortion. Most of the observations reported here were obtained from preparations precipitated with potassium biphthalate buffer at *p*H 4 and stained with 1 to 2 percent uranylacetate at *q*H 5 for 1 5 to 60 minutes before dehydration. All enzymes were used at a concentration of 0.1 mg per ml.

OBSERVATIONS ON BASIC CHROMOSOME STRUCTURE

When the surface-film method is used, chromosome breakage occurs during the spreading action, preventing identification of specific chromosomes or lateral loops. However, long portions of main chromosome axes and associated loops can be obtained on grids (fig. 1). In addition to breaking chromosomes, the surface-film action removes RNP matrix material from loops, providing a gradient ranging from loops which have retained most of their matrix (figs. 1 and 3) to those nearly devoid of matrix material (figs. 2 and 3). Removal of loop matrix reveals that the continuity of each loop is maintained by a thin axial fiber (= 1 chromatid). This fiber is broken by DNase but not by trypsin, pepsin, or RNase. When the matrix is cleanly removed from portions of the loop axis, this fiber shows intermittent thick and thin regions, the thinner regions being more prominent when severe stretching has occurred in the surface film. The diameter of the thicker regions ranges from 75 to 100 A, and the thinnest regions resolved by this technique are 30 to 50 A in diameter.

The thicker portions of the axial fibers apparently consist mainly of protein, since trypsin digestion results in a general decrease in diameter and loss of electron opacity with uranyl staining at *p*H 5.

In surface-film preparations the interchromomeric fiber (= 2 chromatids), which never exhibits RNP matrix, is similar in structure to the loop axes, but, on the average, is slightly wider, ranging from 50 to 75 A in the thinner regions and up to 200 A in diameter in the thicker portions. The chromomeres along the main axis generally are too compact and opaque to permit resolution of internal structure or observation of how loop axes or interchromomeric fibers are inserted. Sometimes, however, chromomeres are partially spread by the surface-film action and show a basic fibrillar unit similar in structure and diameter to denuded loop axes (fig. 5).

In microcentrifugation preparations background debris from nucleoplasm precipitation can be eliminated and better resolution of axial fibers obtained. During centrifugation the interchromomeric fibers often are pulled or stretched until the chromomeres become spaced 2 to 10 μ apart on the main chromosome axis (figs. 4 and 7). Frequently a granule 500 to 700 A in diameter is found on this fiber and usually is located approximately midpoint between adjacent chromomeres (fig. 7). As in surface-film preparations, the interchromomeric threads show alternating thick and thin regions. Before protease treatment, the main axis measures 50 to 150 A in diameter (figs. 8 and 9). After partial trypsin digestion, the thinner regions of the main axis measure 30 to 50 A in diameter (figs. 10 and 11) and the thinner regions in loop axes exposed by digestion of the loop matrix material are 20 to 30 A in diameter. In no instance has doubleness of either loop axes or main axes been observed, even though the latter fiber is known to contain 2 chromatids.

OBSERVATIONS ON THE RIBONUCLEOPROTEIN MATRIX OF LATERAL LOOPS

Three types of matrix material have been observed in centrifuged preparations. One type is found on a large majority of the lateral loops and consists of clusters of ribosome-like particles interconnected by a network of thin fibers (figs. 6 and 12). The basic granular unit appears to be 250 to 300 A in diameter, and in favorable configurations these appear to be spaced 1200 to 1500 A apart on the fibrous component. Granules up to 800 A in diameter are observed in this type of matrix, but these may result from coprecipitation of two to several of the basic granular units. Estimates of the number of granules in single large matrix units from typical loops indicate that these units would be as long as 8 to 10 μ if all the granules in one unit were attached to a single fiber.

A second type of matrix (figs. 15 and 17) can be correlated with the fuzzy loops observed near the middle of chromosome 10 and terminal on one arm of chromosome 4 in light microscope preparations from *Triturus viridescens*.

In this type, the basic matrix unit is composed of long thin fibrils which range up to 20 μ in length when stretched in surface-film preparations (figs. 16 and 20) and up to 10 μ in centrifuged preparations. When these fibrils are stretched in centrifuged preparations, they appear to have granular units similar to those found loosely spaced in typical matrix units. On the fuzzy loops, however, these particles are closely packed on a thin axial fiber (fig. 13). Trypsin removes the granular component from loop matrices (electron microscope observation), whereas RNase breaks the fibrils found on fuzzy loops (phase contrast observation). These observations indicate that the matrix units on lateral loops are composed of an RNA fiber which either has a protein coat along its entire length or has protein granules spaced periodically along its length. The similarity between the granular units of both types of matrix material may be more apparent than real, however, since typical loop matrices precipitate between *p*H 3 and 5 and fuzzy loops precipitate below *p*H 3. A third type of matrix, which has only occasionally been observed, appears somewhat intermediate in structure between the typical and fuzzy-type matrices (fig. 18).

When both thin and thick insertion ends of the same loop can be observed (figs. 19 and 21), the increase in loop diameter from the thin to the thick end appears to be due to an increase in size or length of the matrix unit rather than a change in over-all morphology of the unit. This is especially clear in fuzzy loops, where the matrix fibrils show changes only in length from the thin to the thick insertion end (fig. 15). In addition, there appear to be no abrupt changes in matrix unit size along the loop axis, but rather a gradual transition from a very small cluster to a large cluster on typical loops and from a short fibril to a long fibril on fuzzy loops.

In surface-film preparations in which loop matrices have been partially removed, matrix units at the outer turn of loops are often still attached to the loop axis but partly to completely uncoiled or straightened by the action of the surface film (figs. 16 and 20). When junctures of these matrix units and loop axes can be observed, the points of contact are marked by only a slight thickening along a very short length of the loop axis. In preparations where matrix material has been almost completely removed from long stretches of loop axes, granules ranging from 100 to 700 A in diameter are found on loop axes, as well as in the background precipitation of nucleoplasm. In some cases, several granules (presumably the basal portions of sheared-off matrix fibers) may be attached to the loop axis with a periodicity of about 1000 A (fig. 14).

DISCUSSION

In previous electron microscope studies on lampbrush chromosomes, estimates of the diameter of the interchromomeric fiber (2 chromatids) have ranged from 200 A (*6*) to nearly 1000 A (*7*), whereas the lateral loops

(1 chromatid) have been considered as having a single axis between 200 and 500 A in diameter (*4*) or as composed of a bundle of fibrils, each about 500 A in diameter (*8*). The observations reported here indicate that the minimum diameter of loop axes is 20 to 30 A and that the interchromomeric strands measure 30 to 50 A. These diameters are similar to those obtained by other methods for single double-helix DNA (*9*) or DNP molecules (*10*), or for two double-helices of DNA precipitated together (*11*). These results strongly suggest that the chromatid of lampbrush chromosomes consists in width of a single DNA or DNP molecule. They also corroborate similar conclusions drawn by Gall (*12*) from the kinetics of breakage of loops and main axes of lampbrush chromosomes by DNase.

Labeling with RNA and protein precursors shows immediate incorporation of label into both substances over the entire length of both typical and fuzzy-type loops. Gall and Callan (*13*), however, reported that the giant granular loops on chromosome 12 of *Triturus cristatus* show immediate labeling over the entire loop for protein but sequential labeling from the thin to the thick insertion ends for RNA. They hypothesized that RNA was synthesized at one site near the thin insertion end and then carried around the loop by movement of the DNA axis of the loop. The increase in width of the loop from the thin to the thick end was ascribed to protein synthesis.

Recently, Gall (*14*) broke typical loops by X irradiation before labeling the RNA and found postirradiation incorporation of precursor to occur on both sides of breaks. His results indicate that RNA synthesis is occurring simultaneously along the entire length of typical loops unless one assumes that the breakage itself somehow initiates new sites of RNA synthesis on the thick insertion side of the loop. These results, combined with the observations of matrix fine structure described here, suggest that the synthesis of RNA molecules is initiated repeatedly at the thin insertion end of typical loops and continues around the entire length of a stationary loop axis with each molecule continuously being unzipped from the loop axis as it is synthesized. At any one moment, then, only a very short segment of the newly synthesized portion of each RNA molecule would be in contact with the loop axis while the older portions of the molecules, the oldest region being at the unattached end, would extend into the milieu of the nucleoplasm. The similarity of structure along the length of the RNP molecules on fuzzy-type loops and throughout the clusters on typical loops suggests that the protein component of loop matrices is synthesized on or incorporated onto the RNA molecules at the juncture of the loop axis and newly synthesized portion of each RNA molecule.

The large number of matrix units per loop indicates that a correspondingly high number of RNA polymerase molecules may be present on the loop axes. Counts of units along fuzzy loops, as well as the periodicity of granules sometimes observed on loops stripped of matrix, suggest that the presumed polymerase molecules are spaced approximately 1000 A or less apart along the entire length of each loop axis.

The longest RNP units from fuzzy loops so far measured have been about 20 μ long, whereas the lengths of fuzzy loops floating in solution (*i.e.*, unstretched) range up to 200 μ long. If the postulated mechanism of RNA synthesis is correct, a comparison of the relative lengths of loop product and loop axis indicates that the RNA polymerase molecules may not be reading the entire length of the DNA in the loop axis, or, alternatively, that the length of the loop axis does not consist entirely of DNA. Since DNase digestion appears to disintegrate the entire loop axis, the latter alternative seems unlikely.

Rough estimates of the number of RNP molecules per loop and the average amount of RNA per unit length of DNA in loops can be made as follows. The average length of lateral loops on lampbrush chromosomes of *T. viridescens* has been reported to be 50 μ (*4*). The average length of matrix units is estimated in this study to be about 10 μ, and the spacing of units along the loop axis to be approximately 1000 A. Under these conditions, the number of RNP molecules per average loop would be 500 and there would be 100 times as much RNA, on a length basis, as DNA in the lateral loops.

One is also tempted to speculate on the significance of the length of the RNP fibrils with respect to the function of messenger RNA. Assuming a triplet code and a translation length of 10.5 A per triplet (*15*), an RNA molecule 10 μ in length would be sufficiently long to code for the largest known protein molecules (*e.g.*, glutamic dehydrogenase, MW = 1×10^6). Because most proteins are considerably smaller than this and RNP molecules longer than 10 μ are present on many of the lateral loops of lampbrush chromosomes, it appears that fully transcribed RNA molecules may contain more RNA than required for functioning as templates for single protein molecules. At least three explanations can be suggested: 1) The transcribed RNA remains intact after leaving the chromosome, but either the entire length of the molecule is not used during translation for a single protein or it is used to translate for more than one protein. However, the fact that estimated messenger RNA lengths in the largest polysomes so far observed [2.5 μ (*16*); 7.5 μ (*17*)] still are considerably shorter than the longest RNP molecules appears to argue against this as a general explanation. 2) Each completed molecule of RNA on the chromosome is subdivided into portions that have been transcribed by several structural genes contained within a single loop axis. These subunits of RNA could be separated during transfer to or within the cytoplasm in a manner analogous to the subdivision of nucleolar RNA into ribosomal RNA's reported by Perry (*18*). 3) Each completed RNA molecule on the chromosome may have a subunit that is involved in the movement of messenger portions to sites of protein synthesis but which is detached or degraded before the translation process begins.

RESUMEN

Se describe un método de una película superficial y otro de microcentrifugación para preparar cromosomas plumulados para microscopía electrónica. Los resultados

obtenidos con los cromosomas de *Triturus viridescens* muestran que el diámetro mínimo del eje de los brazos laterales (un cromátide en ancho) digestibles por la desoxiribonucleasa, mide 20 a 30 A° mientras que el filamento intercromomérico del eje cromosómico principal (dos cromátidos en diámetro) digestible por la desoxiribonucleasa mide 30–50 A°. Estos diámetros son similares a aquellos obtenidos por otros métodos para una sola doble hélice de ácido desoxiribonucleico o moléculas de desoxiribonucleoproteína indicando que los cromátidos de estos cromosomas están formados en ancho por una sola molécula de ácido desoxiribonucleico o desoxiribonucleoproteína. En los lazos laterales fueron observados tres tipos de ribonucleoproteína en forma de matriz. El único cambio morfológico que ocurre en la matriz de los lazos es un incremento en su longitud que va desde las inserciones delgadas hacia las inserciones gruesas. Esto sugiere que la síntesis de moléculas de ácido ribonucleico comienza en el extremo delgado de los lazos y continúa a manera de un cierre relámpago alrededor deleje del lazo en toda su longitud.

Las unidades mayores de la matriz son mayores de lo que podría esperarse de la longitud de las moléculas de ácido ribonucleico estimadas por otros métodos. Esto sugiere que las fibrillas de nucleoproteínas de estos cromosomas pueden subdividirse en unidades menores antes de entrar en la síntesis de proteínas. La mayor unidad de matriz observada es mucho más corta que los lazos sugiriendo que la totalidad del ácido desoxiribonucleico en el eje de los lazos, no es utilizado en la transcripción o bien que el eje del lazo no está formado en su totalidad por ácido desoxiribonucleico.

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PLATE 1

FIGURE 1.—Portion of main axis (M) of lampbrush chromosome with chromomeres (C) and associated lateral loops (L). Surface film. × 2,000

FIGURE 2.—Lateral loop denuded of most of ribonucleoprotein matrix material, exposing the loop axis (L). Surface film. × 5,000

FIGURE 3.—Cluster of lateral loops partially stripped of ribonucleoprotein matrix material (MA) with exposed loop axes (L). Surface film. × 3,000

PLATE 2

FIGURE 4.—Main axis of lampbrush chromosome showing interchromomeric fiber (M), chromomeres (C), and lateral loops (L). Opaque clusters in background are peripheral nucleoli. Microcentrifugation. × 2,000

FIGURE 5.—Partially spread chromomere showing fibrils similar in diameter to loop axes. Surface film. × 9,000

FIGURE 6.—Units of typical type of loop matrix that have been separated from loop axes during centrifugation. × 27,000

FIGURE 7.—Main chromosome axis showing granules (*arrows*) which frequently are observed on interchromomeric thread. Alternating thick and thin regions of thread are evident. Microcentrifugation. × 7,000

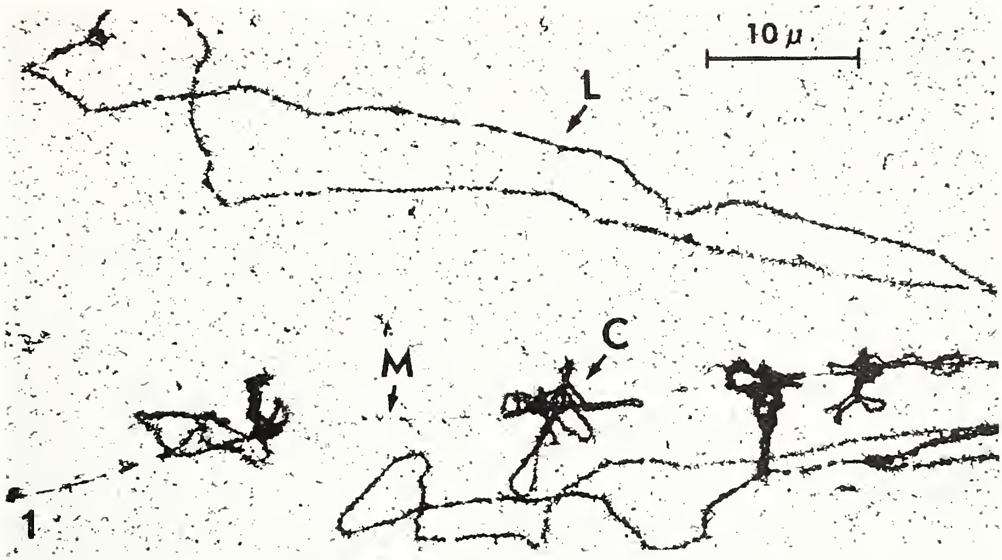
PLATE 3

FIGURE 8.—Main chromosome axis showing interchromomeric thread (*arrow*). Microcentrifugation. × 6,000

FIGURE 9.—High-magnification view of interchromomeric fiber shown in figure 8. Minimum diameter of thread is approximately 50 A. Microcentrifugation. × 37,000

FIGURE 10.—Lampbrush chromosome which has been partially digested with trypsin. Chromomeres (C), interchromomeric thread (M), and loops (L) are designated. Microcentrifugation. × 7,000

FIGURE 11.—High-magnification view of portion of main axis shown in figure 10. Minimum diameter is approximately 30 A. Microcentrifugation. × 162,000



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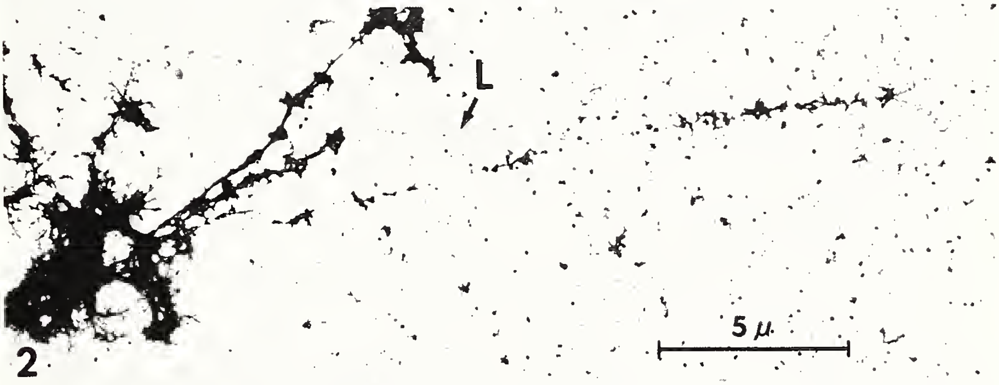

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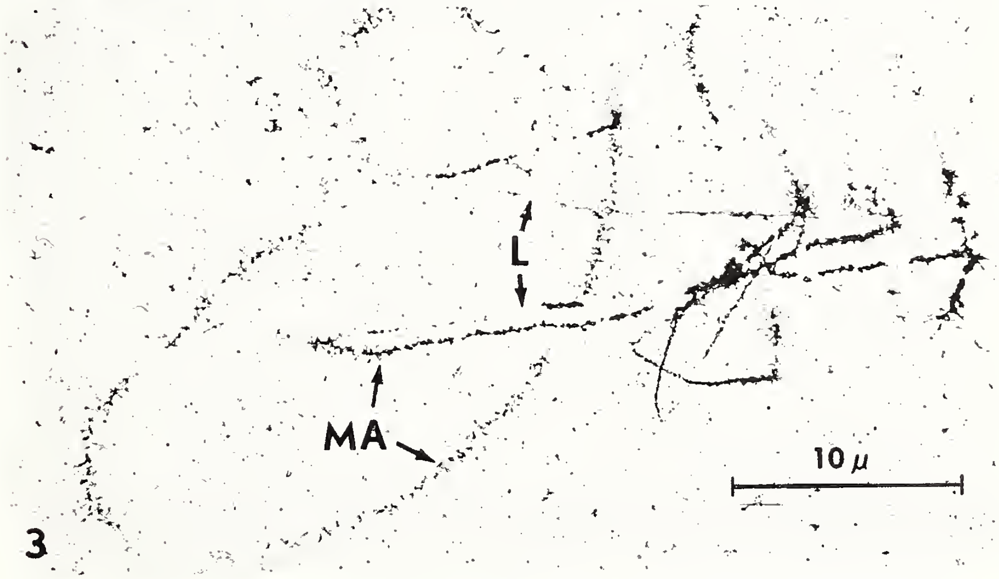

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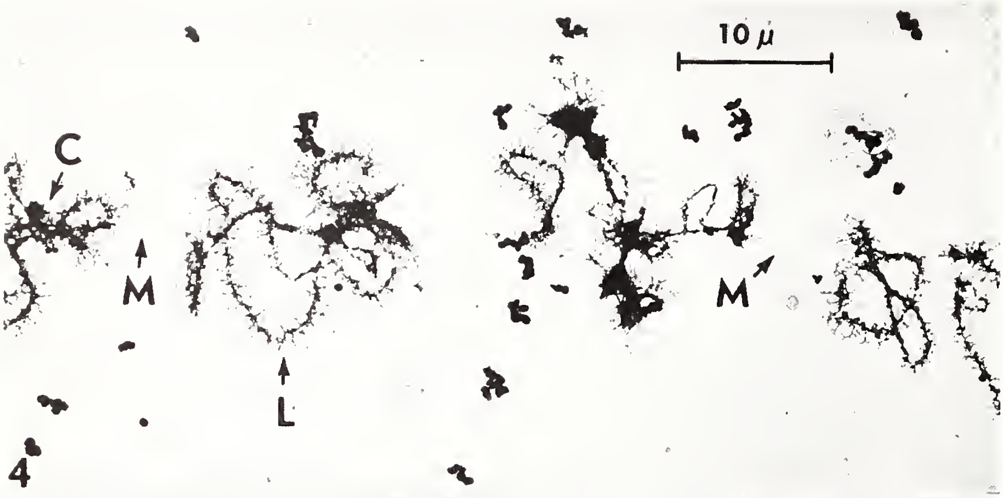

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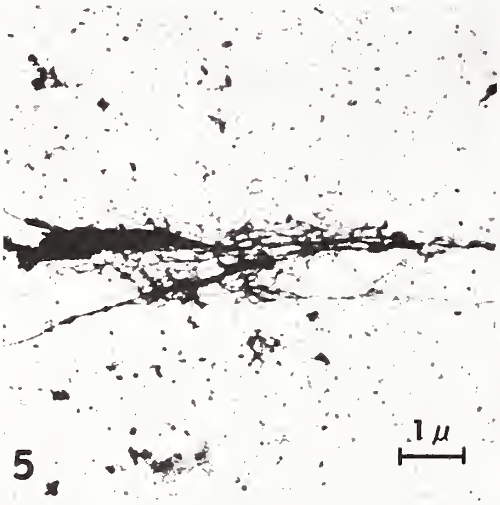

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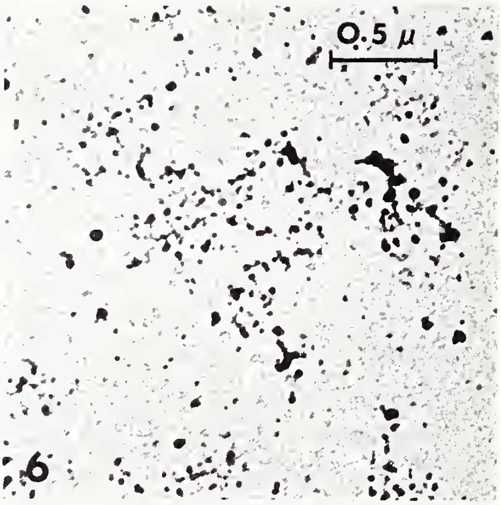

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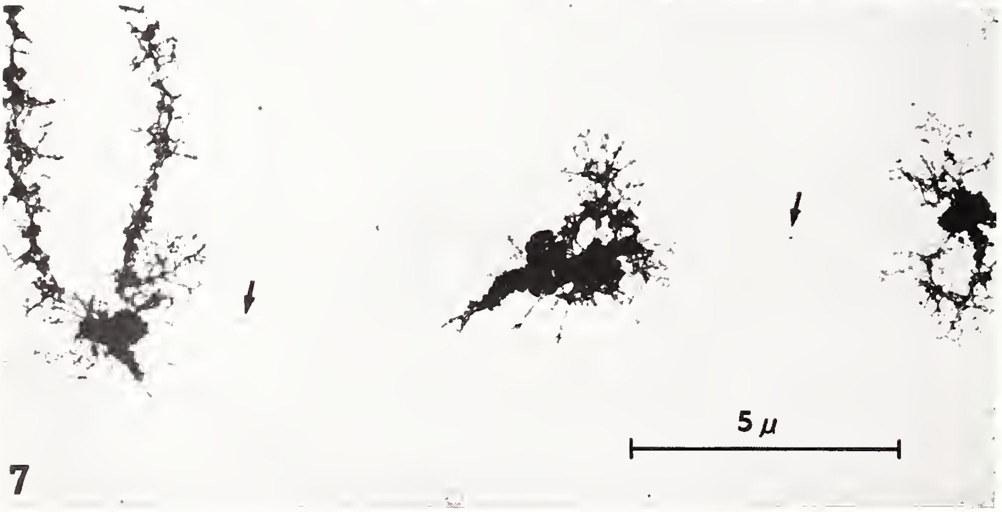

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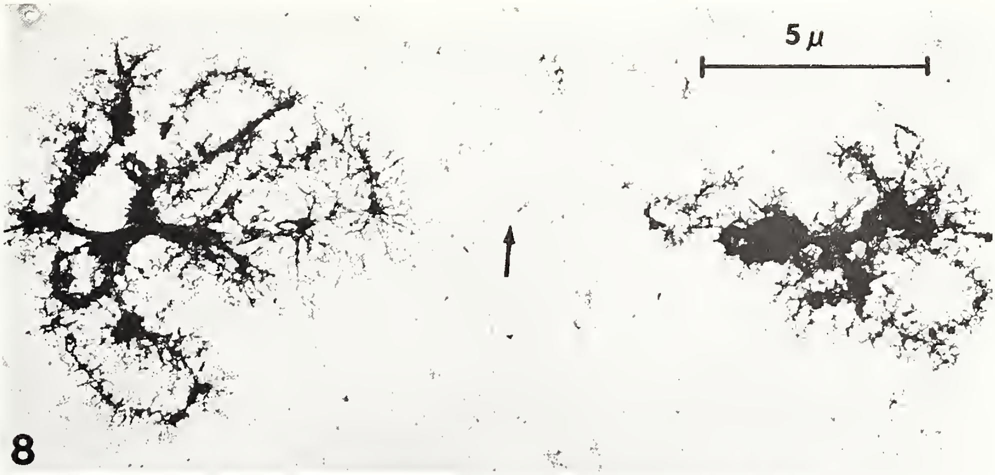

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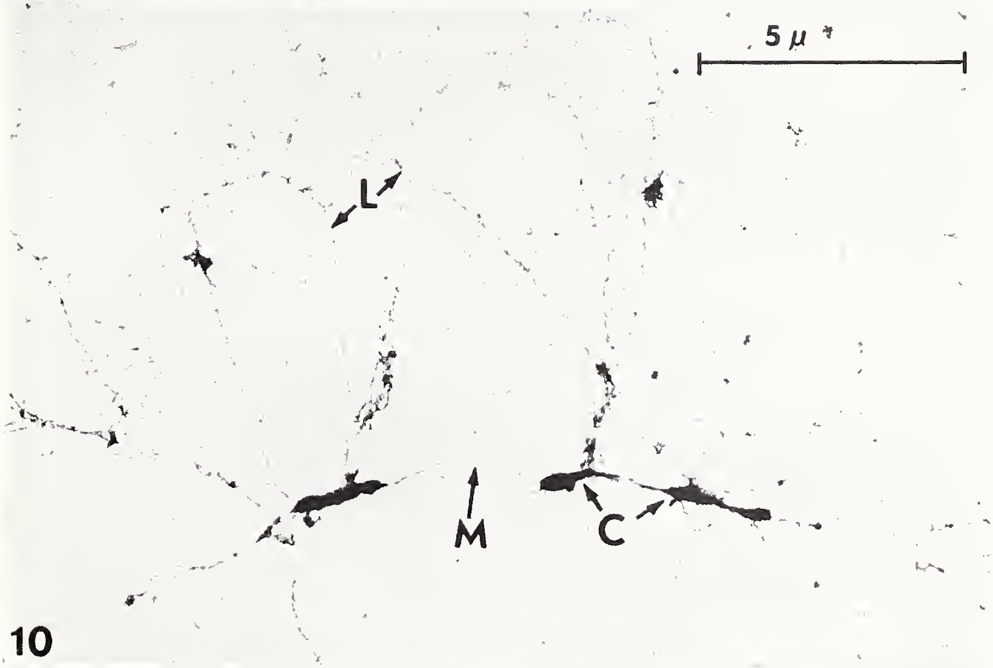

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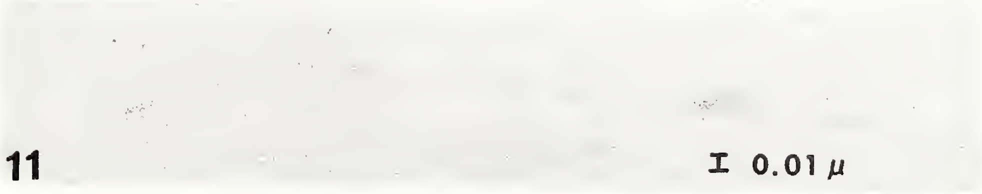

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Plate 4

Figure 12. Lateral loops showing typical type of ribonucleoprotein matrix. The smallest well-defined particles are 250 to 300 A in diameter. Microcentrifugation. × 9,000

Figure 13.—High-magnification view of portion of a microfibril from fuzzy-type loop. Microcentrifugation. × 25,000

Figure 14.—Lateral loop axis stripped of matrix but showing attached granules with a periodicity of about 1,000 A (*arrow*). Surface film. × 11,000

Figure 15.—Fuzzy-type loops with well-defined microfibril as basic matrix unit. The fibrils gradually increase in length from thin insertion side (A) to thick insertion side (B) of loops. Microcentrifugation. × 3,000

Plate 5

Figure 16.—Microfibrils (F) of fuzzy loops uncoiled by surface-film action. Lateral loop axes are designated (L). Surface film. × 6,000

Figure 17.—High-magnification view of portion of fuzzy loops shown in figure 15. Contour lengths of microfibrils range up to 10 μ. Microcentrifugation. × 10,000

Figure 18.—Lateral loop showing a type of ribonucleoprotein matrix intermediate in morphology between typical-type matrix clusters and microfibrils on fuzzy loops. Microcentrifugation. × 4,000

Plate 6

Figure 19.—Lateral loop with typical ribonucleoprotein matrix showing gradual increase in size of matrix units from thin insertion end (A) to thick insertion end (B) of loop. Microcentrifugation. × 6,000

Figure 20.—Lateral loop (L) with ribonucleoprotein matrix units uncoiled by action of surface film. The fibrils (F) range from 4 to 20 μ long. Surface film. × 5,000

Figure 21.—Lateral loop with typical ribonucleoprotein matrix showing transition in matrix size from thin (A) to thick (B) insertion ends. Microcentrifugation. × 5,000

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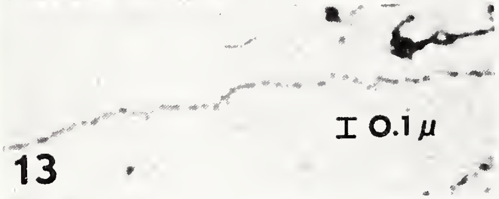
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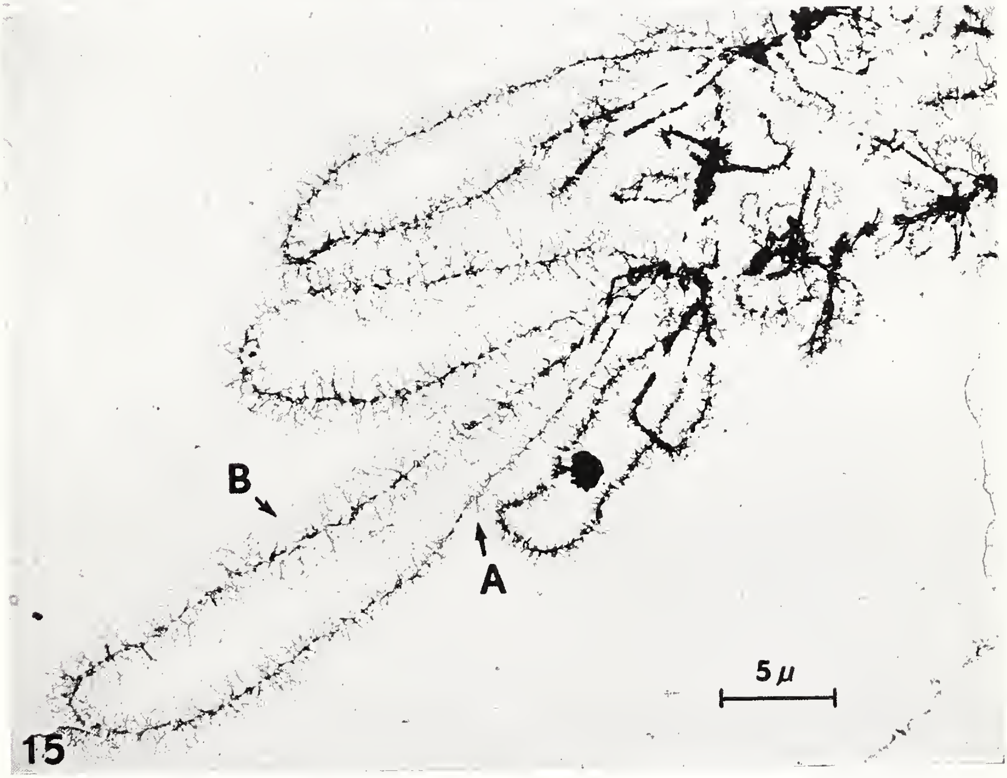
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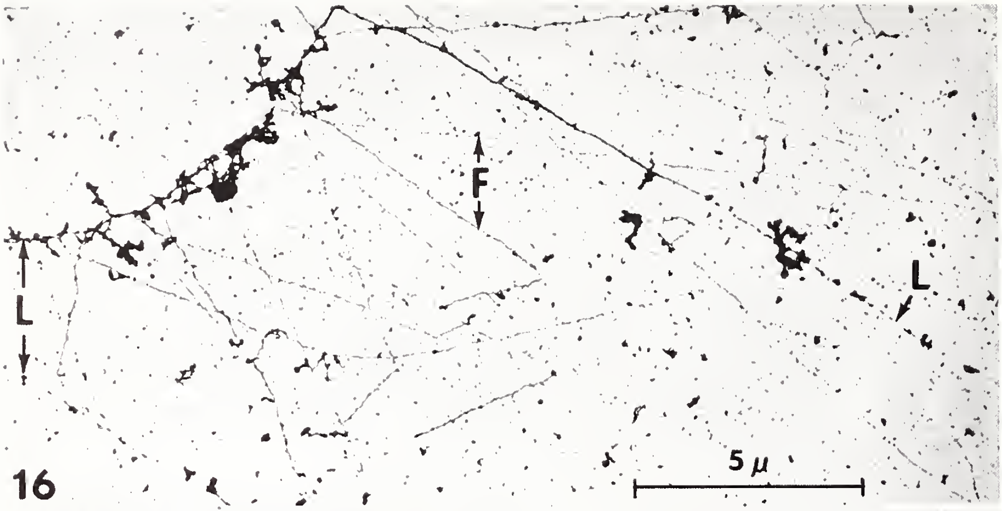

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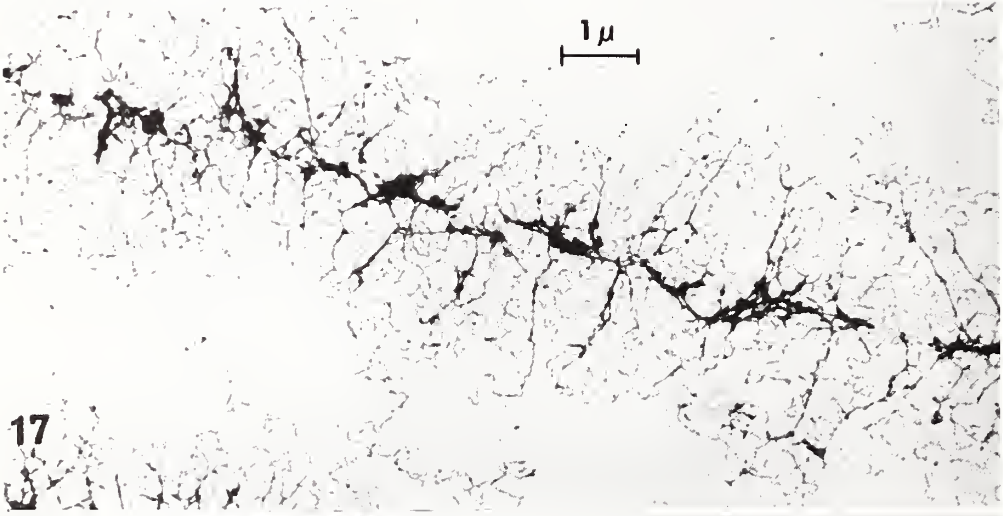

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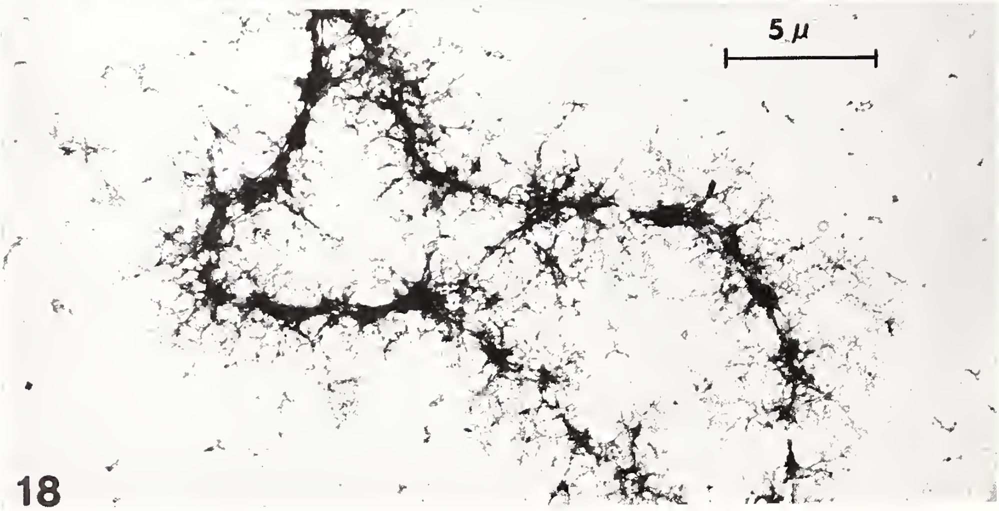

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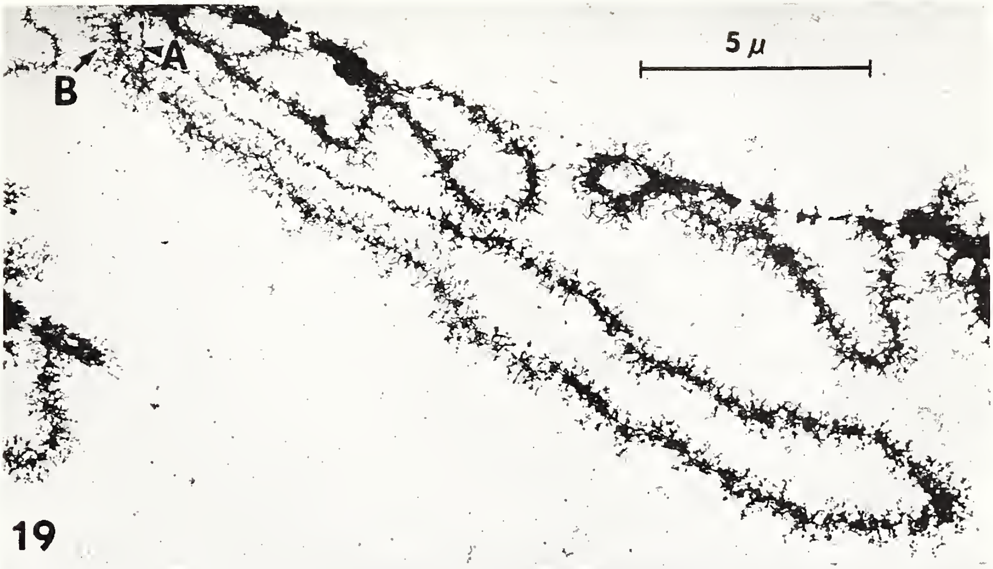

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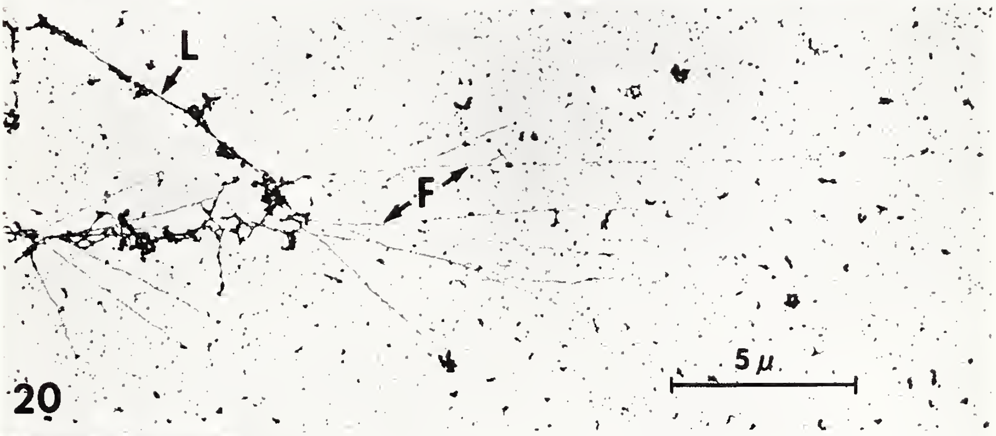

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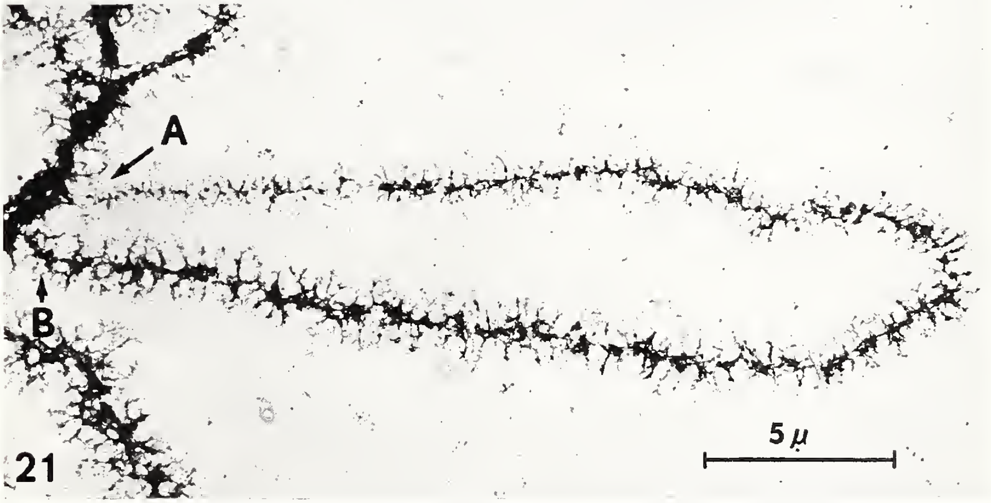

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DISCUSSION

Streisinger: In the diagram you presented at the beginning of your talk, you showed a strand and some wriggles, then a big loop followed by some wriggles and the strand. Your diagram shows a continuous strand and loop that are not closed into a circle. Your photographs always show a big blob where the loop joins the axis. Could there be a circle attached to a strand rather than a loop?

Miller: We cannot follow the axis through a chromomere. This is a conceptual structure based on micromanipulation experiments by Callan, which showed that, when the chromosome is stretched, the chromomeres part in the middle, which indicates a chromomere is not single but double, *i.e.*, it parts between the thin and thick insertion ends of a loop. So it is a circumstantial interpretation. We cannot follow the axis through the chromomere.

Pavan: Please give us some more information about the experiment of Gall in breaking the loops and getting incorporation of uridine on both sides of the broken part. Did you not expect this to happen?

Miller: Gall and Callan, using H^3-uridine, showed sequential incorporation of label from the thin to the thick insertion end of a pair of giant granular loops in *Triturus cristatus*. They interpreted these results as indicating a single site of RNA synthesis near the thin insertion end of the loop. The RNA then was postulated to move around the loop by a movement of the loop axis itself. Gall broke typical loops with X radiation before introducing the label. He found labeling on both sides of the breaks. These results suggest that RNA synthesis in the typical loops occurs along the length of the loop and not at a single site near the thin end.

Lara: Have you made any sedimentation studies with the RNA from these chromosomes?

Miller: Dr. Gall currently is doing this type of work. He has not finished his studies, but I know that he is actively working on this problem.

Lara: We have been working with *Rhynchosciara* which has giant chromosomes and we find that, with short pulses of H^3-uridine, most of the radioactivity is around the chromosome. This is Dr. Pavan's work. In parallel sedimentation studies with extracted RNA, we find a peak of optimal density at 45S, which would correspond to a very high molecular weight RNA like you have found in your work.

Moses: Have you digested any of your preparations with deoxyribonuclease and ascertained whether or not the thin strand, that is, the axis of the loop, is completely broken by the enzyme?

Miller: Yes. The RNP matrix can be partially removed by a variety of methods, for example, detergents, high molarity saline, or low molarity saline. When this is done and deoxyribonuclease is then introduced, these fibers, which are now submicroscopic with phase optics but which can be located by observation of the remaining matrix, are broken by the enzyme.

Moses: You are sure there is no possibility of an elastic fiber still existing after you digested the one visible in the light microscope, *i.e.*, one that would be visible only in the electron microscope?

Miller: It would have to be quite elastic, since the pieces of broken loops may spread several hundred μ apart after breakage by deoxyribonuclease.

Perry: How sure can you be that these strands, which you say are 20 μ long, are one continuous RNA strand? Are you sure of the continuity of RNA? I realize you are sure that the strands are continuous, but they may not consist of single molecules.

Miller: I am not at all sure they are. I said the evidence suggested this, but there is no proof that these are continuous RNA molecules.

Pavan: Did I understand you to say that in the loop you have more than one gene, more than one enzyme-producing unit?

Miller: The polysomes producing hemoglobin consist of 6 to 10 ribosomes and are up to 4000 A long. If one assumes that the length of messenger is near the length of the polysome, then even the largest polysomes now known, which are in polio-infected HeLa cells, with about 60 ribosomes, do not begin to indicate a messenger length of 20 μ. But again I am assuming that the axis of the 20 μ fibers on the lamp-brush chromosomes is all RNA.

Pavan: Could you then relate this type of sequence to that found in microorganisms? This big loop starts from one end and goes through the other end. Could there be something like a combination of 2 or 3 genes, working together in a sequence?

Miller: I am looking at the transcription and not the translation. These are two different things. The actual working of the gene by virtue of the RNA translation occurs mainly in the cytoplasm. I am suggesting that, by this time, the long pieces of RNA found on the chromosome perhaps have broken down into subunits. Another possibility is that there may be some labile RNA in the long pieces involved in the transfer of these molecules to the cytoplasm, but this is pure speculation.

Reissig: Would you care to draw any parallel between these observations and Gall's X-ray experiment?

Miller: I can see no comparison. I forgot to mention in my presentation that Gall's experiment on the kinetics of breakage of these chromosomes by deoxyribonuclease indicates that the axis of loops is a single double-helix DNA molecule in diameter and that the main axis contains two of these molecules. My cytological study confirms what Gall has found by an independent method. But we have no idea whether the RNA polymerase is reading one chain of the double-helix or both. All I am saying is that RNA is synthesized on both sides of the X-ray breaks and this rules out a single site of RNA synthesis in the typical type loop.

Allende: Dr. Spirin has evidence presented during the international meetings that the messenger RNA emerges from the nucleolus, together with some protein in a unit which he calls informosome. Do you have any evidence regarding this?

Miller: No. My evidence is purely cytological.

Schultz: Do you have any way of judging whether there is the formation of new sites of synthesis of these fibers on the matrix? That is, if I understood you correctly, you told us that the matrix fibers grow in length but not in width. But can you tell us whether you can exclude the appearance of new matrix fiber after any particular stage?

Miller: If I understood your question, you are asking if at any particular time a new fiber may start growing at any place along the length of the loop. I have no indication that this happens. Most of the fibers at a certain point along the loop appear to be about the same length, but with this technique I cannot see down into the middle of the loop matrix to rule out short fibers.

Chromosome Replication [1]

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SUMMARY

Replication of chromosomes of higher organisms is discussed from the point of view of the deoxyribonucleic acid (DNA) component of the chromosome. Experiments on DNA in chromosomes of microorganisms are considered in so much as they establish that replication of the DNA molecule, *in vivo*, is semiconservative and sequential. With these properties as basic premises, results of autoradiographic experiments on a number of plants and animals provide a general pattern of semiconservative duplication for the chromosomes of higher organisms. Asynchronous replication along chromosomes demands that several DNA molecules contribute to the longitudinal makeup of the chromosome. The conclusion that a chromosome is constituted by one DNA "piece," where this consists of several molecules linked in an end-to-end fashion, is challenged on the basis of exceptions to the general autoradiographic pattern. Isolabeling, labeling over homologous regions of sister chromatids two divisions after label application, seems to occur widely. An explanation for isolabeling within the framework of one DNA-piece chromosomes necessitates many sister chromatid exchanges over short regions of the metaphase chromosome: The distribution of breakpoints for exchanges does not support this hypothesis. The only alternative considered likely is that there is more than one DNA piece, laterally, in a chromosome. Evidence in support of this assumption is provided by many cytological observations of half-chromatids, both in fixed and living cells. Functional reality of half-chromatids is seen in X-ray-induced aberrations where half-chromatids act as the unit of reunion. It is concluded that a case exists for there being, in *Vicia faba* and perhaps more generally, more than one DNA piece per chromosome. Some mention is made of the finding of apparently circular DNA molecules in the nuclei of salamanders and newts. In the Plethodontid salamanders there is evidence that these "ring nucleoli" are produced at a specific locus on one chromosome. The possibility is discussed that circular DNA molecules may be a regular feature of chromosomes of higher organisms.—Nat Cancer Inst Monogr 18: 101-131, 1965.

INTRODUCTION—Although the genetic function and general characteristics of chromosomes have been known since the beginning of this century, little attention was paid to chromosome structure until about

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

30 years ago. A number of cytologists then claimed that the anaphase chromosome or metaphase chromatid had discernible substructure in many organisms (*1*, *2*). Disagreement on this point was voiced but in the discussions little thought was given to the transition of the anaphase chromosome to the bichromatid metaphase chromosome of the next cell generation. Darlington (*3*) did write in nonspecific terms of chromosome splitting and considered the relationship of relic coiling in prophase to the coiling of the chromosomes of the previous division. More precise statements awaited the establishment of deoxyribonucleic acid (DNA), a major component of the chromosome, as the primary genetic material.

A major contribution in the study of chromosome replication in higher organisms was made by Taylor, Woods, and Hughes (*4*) in their autoradiographic analysis of the cellular inheritance pattern of DNA in the chromosomes of *Vicia faba*. These workers followed DNA distribution by supplying tritium-labeled thymidine to the cells for a given period and then allowing these cells to proceed in their mitotic cycles for varying periods of time before examining them. This isotope, by virtue of the limited range of the emitted beta particles in sensitive emulsion, was better suited to analysis at the chromosome level than was the C^{14}-thymidine used earlier by Plaut and Mazia (*5*). These studies served to stimulate a now widely diversified field of investigations into problems of chromosome replication, structure, and function.

Several excellent reviews have appeared in recent years (*6–10*), and with this in mind I have attempted in the present paper not to discuss the whole of this considerable body of work but rather to present a personal view of the present status of some facets of the problem, principally with regard to the arrangement and distribution of DNA within the chromosome. In doing this I have briefly considered some of the findings on the genetic material of bacteria and phage in order to evaluate some premises basic to arguments concerning chromosomes of higher organisms. I have summarized the results and conclusions drawn from autoradiographic experiments in various plants and animals and have considered these findings in relation to other forms of analysis.

CHROMOSOMES OF BACTERIA AND PHAGE

The application of H^3-thymidine autoradiography to the DNA of microorganisms has enabled Cairns (*11*, *12*) to make some convincing pronouncements on the physical nature of the organization of DNA in *Escherichia coli* and in the T2 bacteriophage. Cairns prepared autoradiographs of DNA from lysed *E. coli* after the cells had grown for two generations in medium containing H^3-thymidine. He concluded that all his results were compatible with the assumptions that the DNA of *E. coli* forms a circular chromosome, and that replication of this chromosome proceeds while the structural circularity is maintained. Comparison of the length of DNA, detected in the autoradiographs with the molecular weight of the total DNA content, leaves little doubt that the chromosome is composed of a single "piece" of DNA, *i.e.*, it is one double-helix molecule wide. Cairns cautions that his autoradiographs do not exclude the possibility that the DNA piece is actually several molecules joined together and, in fact, he proposes the formal necessity for at least one discontinuity in order that chromosome replication be possible. Cairns was also able to conclude that replication of the chromosome was semiconservative and proceeded in one direction from the point of initiation. This cytological demonstration of sequential replication is in agreement with genetic studies by Nagata (*13*) and with comparable work on *Bacillus subtilis* by Yoshikawa and Sueoka (*14*).

The inference of a semiconservative scheme of replication was made by Cairns on the basis of grain counts on the component segments of a duplicating chromosome. Forro and Wertheimer (*15*) also concluded, on the basis of tritium autoradiography, that the *E. coli* chromosome contained two subunits which generally remained intact during successive cycles of replication. These data complement the findings of Meselson and Stahl (*16*) who used the technique of density gradient centrifugation to follow the distribution of heavy nitrogen-labeled DNA in *E. coli*. Their experiments showed that all of the DNA in a bacterium replicates in a semiconservative fashion. The term "semiconservative" is used here according to the proposals of Delbrück and Stent (*17*) and implies that the DNA molecule is composed of two longitudinal units, each of which remains intact and each of which becomes associated with a complement in the process of replication such that two identical daughter molecules are formed. Meselson and Stahl could not identify the conserved unit as necessarily being a single polynucleotide chain, and later Cavalieri and Rosenberg (*18*) proposed that the unit corresponded to a double-helix DNA molecule with the chromosome then being two such molecules. However, joint consideration of the data from Cairns and Meselson and Stahl weighs heavily against the proposals of Cavalieri *et al.* Electron microscope studies of *E. coli* DNA spread on a protein monolayer at an air-water interphase also strongly favor the interpretation of the chromosome as a single piece of DNA of Watson-Crick (*19*) dimensions (*20*). The single DNA-piece construction of the chromosome appears also to be the case in a number of bacteriophages—both where the chromosome is structurally circular or linear (*21–23*).

In short, three properties of the *E. coli* chromosome, emerging from genetic, autoradiographic, and electron microscope investigations, seem to be pertinent here: The chromosome is a single, continuous or discontinuous, piece of DNA composed in all probability of two complementary polynucleotide chains; replication is semiconservative and unidirectional from a single point of initiation. The question may now be asked as to the significance of these findings to the chromosomes of higher organisms. Ris and Chandler (*22*) have recently stressed the differences in levels of complexity of the genetic systems of "prokaryotes" (as typified by bacteria and viruses) and "eukaryotes" (organisms with an *organized* nucleus), but the fact remains that DNA is common to all these systems. Can the molecular properties of DNA ascertained with microorganisms be validly applied to DNA of higher organisms? One point in favor of such extrapolation is that the pathway of synthesis of calf thymus DNA is essentially similar to that of *E. coli* DNA (*see* review by Bessman *24*). Furthermore, a semiconservative replication for mammalian DNA has been demonstrated by Simon (*25*) using techniques similar to those of Meselson and Stahl. It would seem then that in all probability replication of the DNA molecule will follow the same rules in higher organisms as in bacteria; we will assume in the following discussion that DNA molecules in the chromosomes of higher plants and animals replicate semiconservatively and sequentially.

CHROMOSOME REPLICATION IN HIGHER ORGANISMS

A General Pattern

The use of isotopically labeled DNA precursors permitted the delimitation of a specific period of the cell cycle during which the nuclear DNA content was doubled. Taylor and McMaster's (*26*) investigation in *Lilium* provides a representative study of this type: These workers correlated P^{32} incorporation with DNA doubling for both the premeiotic and first pollen grain mitotic interphases. Studies, *e.g.*, Mitra (*27*), on patterns of X-ray-induced aberrations over the cell cycle provided confirmation that the doubling of nuclear DNA correlated with actual chromosome duplication: The change from chromosome to chromatid errors occurred about the time of DNA synthesis. This relationship has recently been demonstrated at a far more sophisticated level by combined X-ray and autoradiographic analyses (*28, 29*). In these experiments it was shown that, in cells containing both chromatid and chromosome errors, the chromosome errors occurred only in unreplicated regions.

The next step was to follow the distribution of newly incorporated DNA in the chromosome and hence determine the mode of replication. The first attempt at providing an answer to this question was that of Plaut and Mazia (*5*). Their observations, with C^{14} autoradiography, were made at the level of whole anaphase nuclei: The results did not permit a conclusive decision as to the type of replication process but the data did not lend full support to a semiconservative scheme. The choice of tritium as the isotope permitted Taylor *et al.* (*4*) to increase the resolution of the autoradiographic technique such that analysis was possible at the level of single chromatids. *Vicia faba*, the organism used in the initial experiments, has 6 pairs of large chromosomes: One pair is metacentric (M) with a prominent nucleolus organizer, and the other 5 pairs are subtelocentric (S) and are not readily distinguished one from the other (*see* fig. 1). Taylor *et al.* employed a simple experimental design in which the distribution of label was examined over chromosomes in metaphases of the first and second divisions after the application of isotope. Tritiated thymidine was made available in the culture medium for a time period approximating one replication period and after removal from this medium the beans were placed in a colchicine solution, which ensured a high frequency of metaphases for analysis and also provided a marker, a tetraploid chromosome complement, for second division cells.

The results obtained were striking: All chromosomes in X_1 (first division after label) cells were labeled equally in sister chromatids, this rule applying even where only a segment of a chromosome was labeled, whereas in X_2 cells all chromosomes showed all-or-none segregation of label between sister chromatids. In some X_2 chromosomes the label was not segregated such that it was all on one chromatid and none on the other, but was distributed over both chromatids. However, in the latter cases the distribution was always in a complementary pattern such that homologous regions of sister chromatids demonstrated label segregation. Taylor *et al.* postulated that in these cases an exchange between sister chromatids had taken place. Cells showing the various labeling patterns discussed are shown in figures 2 to 5. These experiments argued against both dispersive and conservative schemes for chromosome replication and provided strong support to the interpretation presented by Taylor *et al.* They suggested that each chromatid was composed of two complementary subunits which remained intact (except at points of sister chromatid exchange) and became associated with a new complement at the time of replication. This semiconservative scheme was, of course, highly analogous to the mode of replication of a DNA molecule (*see* previous section) and it was tempting to think of the chromatid as being just one "piece" of DNA. However, Taylor *et al.* noted that appropriate segregation from a multistranded structure could yield similar results.

The same general pattern of isotope distribution has subsequently been found in many other organisms, both plants and animals [*e.g.*, *Bellevalia* (*30*); *Crepis* (*31*); *Tradescantia* (Wimber, personal communication); *Allium* (*32*); Chinese hamster (*33*, *34*); human cells (*34*, *35*); and *Potorus*, a marsupial (*36*)].

Properties of the Proposed Subunits

Taylor (*30*) sought further information as to the nature of the hypothesized units by studying patterns of sister chromatid exchanges in *Bellevalia*. He frequently found that the two pairs of homologous chromosomes in tetraploid X_2 cells both had an exchange at the same position along the chromosome; he cogently argued that these "twin" exchanges would result from a single exchange event occurring at the X_1 division. An exchange event occurring in the X_2 division would yield only "single" exchange patterns, *i.e.*, affecting only one pair of homologues. Taylor considered the expected frequencies of twin and single exchanges firstly where the broken subunits of any chromatid were given equal probabilities of reunion with any other broken ends, either in the same or the sister chromatid; and secondly where subunits were assumed to be differentiated such that rejoining of each was restricted to ends of "like" subunits. With the underlying assumptions that the probability of an exchange was equal in the two interphases under consideration and that all breaks involved the two subunits of a chromatid, the ratio of twin to single exchanges on the model of unrestricted rejoining was 1:10 and the corresponding expectation for restricted reunion was 1:2. The assumption of simultaneous breakage of the two subunits followed from the observed absence of chromosomes showing a segregation of label in the X_1—such chromosomes would be expected if breakage-reunion were at the one subunit level. The data obtained, 81 twins to 30 singles, certainly favored the postulate of restricted reunion of some units, but indicated that the probability of breakage was greater in the first interphase than in the second. Subsequently Taylor (*37*) presented data for a particular chromosome of *Bellevalia* in which he showed that the presence of colchicine at the first interphase, as well as at the second, modified the exchange distribution such that twins now approximated one half the frequency of singles. The inference is that colchicine plays a role in the determination of prospect of exchange and it would also seem that the frequency of exchanges is insensitive to the twofold change in amount of tritium per chromosome.

The conclusion emerging from these experiments was that the two subunits of a chromatid were not identical with respect to reunion of broken ends. This property of polarity of subunits heightened the analogy of the chromosome replication scheme to that of the DNA molecule, and Taylor (*9, 10*) has concluded that his autoradiographic experiments favor the interpretation of chromatids being composed of one DNA molecule, or several joined linearly, at the expense of a scheme involving lateral multiplicity of molecules.

Asynchrony of Label

One feature of chromosome replication which has received considerable attention is that of sequence of labeling. In the earlier experiments it was obvious not all chromosomes in a nucleus were synchronous in their initiation and completion of duplication, and it was soon evident that recognizable patterns of asynchrony also existed along individual chromosomes. In *Crepis*, Taylor (*31*) claimed that synthesis was progressive from the ends of the chromosomes toward the centromere; a similar pattern was suggested for *Scilla* and *Fritillaria* (*38*). On the other hand, Wimber (*39*) found in *Tradescantia* that the ends of chromosomes continued labeling after other regions had completed duplication. These terminal segments in *Tradescantia* appear not to be cytologically differentiated, and in this respect the species differs from many others in which late labeling is associated with heterochromatic regions. For example, this seems to be true in such widely diversified organisms as *Vicia* (*40, 41*), grasshopper (*42*), and many mammals. The most striking pattern of asynchrony in mammals is associated with the heterochromatic-euchromatic properties of sex chromosomes. Numerous reports, in many different animals, have confirmed that the heterochromatic X chromosome in the female complement labels at a later time than its euchromatic homologue (*cf 43, 44*). One of the most complex cases, but one which still fits the same rule for sex chromatin, is that reported by Taylor (*33*) for the Chinese hamster: In the female one whole X and the long arm of the other X are late replicating, whereas in the male, the Y chromosome and the long arm of the X are late replicating. This pattern agrees well with the distribution of euchromatin-heterochromatin described for the golden hamster by Ohno, Becak, and Becak (*45*).

The possible significance of this pattern of asynchrony in the mammalian sex chromosomes to the Lyon (*46, 47*) hypothesis for gene action and dosage compensation has been much discussed, but the underlying reasons for the various specific patterns of asynchrony, whether or not it is correlated with heterochromatin-euchromatin distribution, remain obscure. The essential point for the present discussion is that in the course of many of these investigations it has been found that DNA replication may be initiated at more than one point along a chromosome at any given time [*see*, for example (*33, 41, 43*)]. In *Vicia* we have noted, in a pulse-label experiment, as many as three regions of replication along a single chromosome arm. An assumption of many sites of simultaneous replication would seem necessary to explain the rapid labeling of complete chromosomes observed in many of the late-labeling X chromosomes in mammalian cells (*cf 43*). On the premise that a DNA molecule replicates sequentially from a given starting point, the inference must be made that there are several separate molecules along a chromosome.

Requirements for a Chromosome

From the findings mentioned, a chromosome may be envisaged, as far as its DNA component is concerned, as being composed of a "piece" of DNA that consists of a number of double-helix molecules joined together by a sequence of some form of link. A number of models have been suggested, but perhaps the simplest one conforming to these requirements is that proposed by Freese (*48*) and later elaborated by Taylor (*10*). A unineme (single piece) concept of the chromosome is satisfying in that it provides a direct link with the chromosomes of bacteria and viruses and more importantly it is compatible with most genetic data, both from recombination and mutation studies. But, such a scheme would seem to be at variance with much cytological data that suggest a chromatid may have more than one piece or a "strand" in its makeup. There is also some evidence that the autoradiographic patterns may not always be as straightforward as has been indicated in the preceding discussion. A consideration of these points follows.

Exceptions to the General Pattern

Some doubt as to the simplicity of the process of chromosome duplication arose from autoradiographic experiments of La Cour and Pelc (*40*).

They claimed that in *Vicia* not all chromosomes showed label segregation at the X_2 division, and since colchicine had been omitted in their experimental design, they suggested that their results differed from Taylor *et al.* perhaps because colchicine may have influenced the distribution of newly synthesized DNA. Accordingly, they included colchicine during the labeling replication period and reported that now segregation of label was evident at the X_1 metaphase. These experiments were questioned by Woods and Schairer (*49*) who presented data demonstrating that grain distributions over sister chromatids at X_1 satisfied a one-to-one expectation whether the colchicine had been present or not.

Other results not in complete agreement with those of Taylor *et al.* were obtained by the present author (*50*). This series of experiments with *Vicia* were of the same general design as those of Taylor *et al.*, but colchicine treatments were varied so that some evaluation of the two proposed colchicine effects could be obtained: These were 1) La Cour and Pelc's claim that colchicine modified normal chromosome duplication and 2) Taylor's finding in *Bellevalia* that colchicine reduced the probability of sister chromatid exchange. The experiment provided no support for the first proposition in that results obtained for both the X_1 and X_2 divisions were qualitatively uniform in treatments in which colchicine was present in the first and/or the second replication, or in which it was omitted completely. This consistency held on a statistical basis for experiments in which quantitative data were collected. At the X_1 metaphase, chromosomes showed equal labeling over sister chromatids as had been reported by Taylor *et al.* Occasional chromosomes did show a segregation of label (*see arrow* in fig. 2) but the presence of colchicine appeared not to increase their frequency to any appreciable degree. The random nature of decay and production of the latent image make it difficult to attach significance to these cases, and, in fact, statistical tests of grain distribution [(*37*, *49*); Peacock, unpublished data] have revealed no significant asymmetry between chromatids. These analyses do not exclude the possibility that some of these chromosomes represent actual segregation of label, but they show that at grain densities achievable at present this possibility is not resolvable. On the other hand, Taylor (*10*) reported that he has detected significant asymmetry with high specific activity H^3-thymidine and has interpreted this to be evidence for out-of-phase replication of the two subunits of a chromatid.

The exceptions to the expected labeling pattern were obtained in the X_2 divisions. Although most chromosomes exhibited a clear segregation of isotope, with or without sister chromatid exchanges, a significant proportion (241/1164) displayed silver grains over homologous regions of sister chromatids: This condition was termed isolabeling. Isolabeling was sometimes restricted to a region of a chromosome which elsewhere had label segregation, but in other cases appeared to extend along the full length. Segmental isolabeling was most frequently found to be terminal (figs. 8 and 9), but isolabeled segments also occurred in between regions of label segregation (17 examples of intercalary isolabeling in 241 isolabeled chromosomes—*see* figs. 6, 7, and 8). One other feature of interest arising from these experiments was that the data gave no indication that colchicine modified the prospects of sister chromatid exchange: There were no significant differences between the over-all means or the frequency distributions of exchanges for either the M or S chromosomes in the different treatments.

Sister Chromatid Exchange

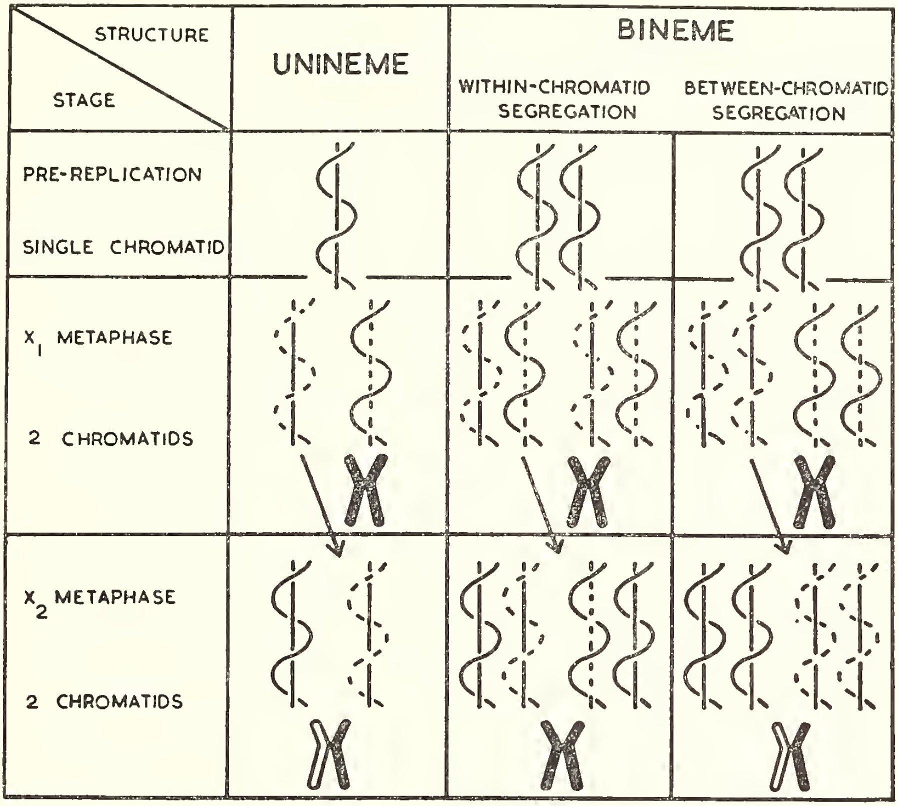
Taylor showed in *Bellevalia* that the frequency of sister chromatid exchange was proportional to chromosome length and this relationship was confirmed by Peacock for *Vicia.* Little else is known about these events. Taylor (*10*) has claimed that sister chromatid exchanges supply "unmistakable visual evidence" for events similar to the spontaneous exchanges occurring in ring chromosomes of maize. However, since Wimber (*51*) first showed a dependence of chromosome breakage on levels of incorporated H^3-thymidine, the possibility has existed that the exchanges are in reality an artifact following breakage induced by tritium decay. Wolff (*52*) has recently pointed out the ratio of one single exchange to two twin exchanges, which Taylor obtained in *Bellevalia,* is exactly what would be expected for a two-hit error. Taylor found that this ratio could be modified by varying colchicine treatment until it approached the ratio of two singles to one twin expected on his hypothesis of spontaneous origin. But the data on this point are few and since Peacock found no evidence for the colchicine effect in *Vicia,* the meaning of this effect is uncertain. Marin and Prescott (*53*) scored sister chromatid exchanges in Chinese hamster chromosomes and found no significant change in frequencies over a 100-fold change in level of incorporated tritium, whereas increases in X-ray-induced errors were detected over a small dose range. Their conclusion, that the majority of sister chromatid exchanges are spontaneous, assumes, as Wolff has pointed out, that a saturation of exchange sites was not achieved by the lowest tritium level. If the exchanges are radiation induced, then it seems surprising that U-shaped reunions or translocations do not occur with considerable frequency. In the 634 chromosomes having one or more sister chromatid exchanges in the *Vicia* experiments (*50*), none were involved in translocations or had U-shaped reunions, although 220 had two or more exchanges. A rejoining pattern of this degree of nonrandomness could be a property of a natural system of exchange, but it would be unprecedented for radiation-induced breakage. In view of these considerations, all that can be safely concluded about sister chromatid exchange on the basis of present data is that its nature remains an enigma.

Significance of Isolabeling

Isolabeling in the X_2 division has now been reported in organisms other than *Vicia:* Walen (personal communication) finds it in a considerable frequency in cultured cells of *Potorus,* a marsupial, and Evans (personal communication) has examples in *Allium*. Taylor (*30*) did find such chromosomes in *Bellevalia*, but in very low frequencies. The question now arises as to the origin of isolabeling. The possibility of incorporation during the second interphase from a residual pool of isotope can certainly be discarded for *Vicia*. Checks by Evans (*41*) and Peacock (unpublished) have established that, over the concentration ranges used in these experiments, the supply of H^3-thymidine is not detectable a few minutes after the roots have been removed from the isotope. Peacock used a chase of cold thymidine in his experiments, and Evans has shown that the use of a chase extends the period of availability of the isotope but still not within the time limits permitting utilization in the succeeding interphase. Taylor suggested that segmental isolabeling resulted from interchromosomal exchange in the colchicine tetraploid cells he was examining. Wolff (*52*) furthered Taylor's explanation for isolabeling by suggesting that, since a measurable degree of somatic pairing is claimed in *Vicia* (*54*), then exchanges between sister homologues might represent the origin of isolabeled segments. On this scheme there would be a corresponding "iso-unlabeled" region on the other homologue. Absence of label provides a poor point of argument in autoradiographic work, but, on the other hand, this postulate can certainly be rejected, on positive grounds, for at least some cases of isolabeling because of the absence of appropriate iso-unlabeling (*see* the M chromosomes in fig. 8).

A third explanation for isolabeling is that it is an artifact introduced by the limits of resolution of the technique. If there were a number of sister chromatid exchanges over a small region of the metaphase chromosome, then the autoradiograph would yield grains over apparently homologous regions. The only argument that can be offered here is that in *Vicia* the distribution of exchanges provides a satisfactory fit to the Poisson expectation, with the probability of exchange being less than one per chromosome arm (0.699 for S chromosomes, 0.554 and 0.629 for the arms of the M chromosomes). Nothing in this data suggests that some regions would have many exchanges, nor do grain counts indicate a correlation of isolabeling with high regional concentrations of isotope. Thus, although this multiple exchange postulate cannot be completely excluded, present data offer it no support, particularly when considered with the lack of any *a priori* reason for expecting such exchanges, whether they are considered to be produced by radiation damage or by a spontaneous method.

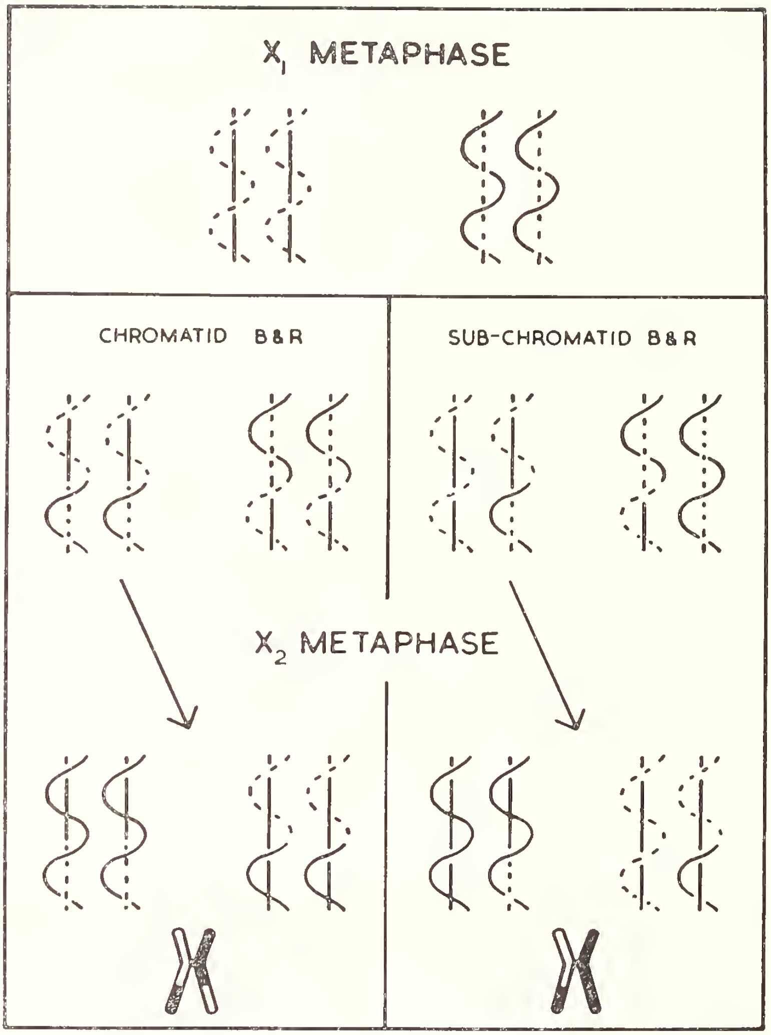
Another way to account for isolabeling rests on the supposition that there is more than one piece of DNA per chromatid, *i.e.*, a lateral multiplicity. Text-figure 1 illustrates that, with a minimum assumption of two DNA molecules (a bineme), appropriate segregation of the conserved single polynucleotide chains can yield either label segregation or isolabel at the X_2: In contrast to this a unineme produces only label segregation. In the diagram the chromosome is considered lengthwise as a single DNA molecule—the introduction of links would not alter the label distribution pattern. The necessity of segregation of sister single helices, one to each



Text-figure 1.—Possible distributions of DNA helices in chromosomes having one or two "pieces" of DNA. Presence of isotope is indicated by a *broken line* in DNA helices and by blocked-in chromatids. [Reprinted from Peacock (*50*).]

daughter chromatid, for the generation of label segregation was first set down by Steffensen (*6*). Isolabeling results from a breakdown of the semiconservative segregation scheme. Segmental isolabeling can also be accommodated with a multistranded chromosome: In text-figure 2, terminal segmental isolabeling is shown as a possible result if DNA "pieces" were to act as the unit of reunion after a breakage event. On this basis, intercalary segmental isolabeling arising from two exchanges would be expected in lower frequency than the single event terminal isolabel, as indeed is the case.

Thus, isolabeling is regarded to provide evidence that a single DNA piece scheme for the *Vicia faba* chromosome is insufficient; at least two DNA pieces per chromatid are demanded. If grain densities of a higher order could be obtained, then a comparison of counts for sister chromatids in isolabeled regions could conceivably yield an estimate of the minimum number of DNA molecules present, *e.g.*, a 3:1 distribution would necessitate four molecules per chromatid. It should also be noted that asynchronous replication between the component molecules in a multistranded



Text-figure 2.—Sister chromatid exchange and segmental isolabeling arising from different patterns of reunion following a breakage event. Presence of isotope is indicated by a *broken line* in DNA helices and by blocked-in chromatids. B and R = breakage and reunion. [Reprinted from Peacock (*50*).]

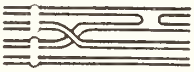
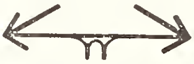
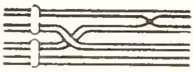
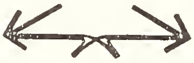
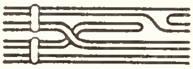
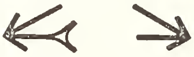
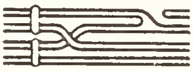
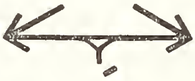
chromosome could yield label segregation or inequality of label at the X_1 metaphase. Both of these conditions have been observed (*10, 40, 50*) and are difficult to reconcile with a single DNA molecule scheme.

Other Evidence for Multistranded Chromosomes

Cytologists have frequently claimed to have seen half-chromatids and, despite criticisms which maintain the "strands" result from poor fixation or optical artifact, some of the observations seem indisputable, *e.g.*, Manton's (*55*) photographs of *Todea* chromosomes using the ultraviolet microscope. Half-chromatids are readily seen in figure 1 and in many of the chromosomes the number of gyres of relational coiling between these strands can be determined. Confirmation of the reality of these structures is given by Bajer's observations on living cells in *Haemanthus*. Bajer has films which show in normal anaphase cells half-chromatids similar in appearance to those in figure 1; he also could demonstrate substructure in the chromatid at sites of neocentric activity and following ultraviolet-microbeam irradiation of the centromere region [Bajer (*56*), and personal communication]. Wolff, in this Symposium, describes experiments performed by Trosko on isolated *Vicia* chromosomes in which trypsin treatments have revealed half- and perhaps quarter-chromatid structures. Hughes-Schrader (*57*) has reported that in the scale insect, *Llaveiella*, half-chromatids frequently proceed independently to the poles at anaphase. A number of lines of inferential evidence support a multistranded chromosome (*see* reviews cited in Introduction). Differing chromosome sizes in closely related species are considered an indication of polyneme differences. DNA measurements in related coccid species offer experimental support to this concept (*58*). Similar evidence is at hand for the plant genus *Luzula* (*59*). Rudkin (*60*) has shown what must be polyneme differences in cells of *Drosophila:* He showed that in some cells the amount of DNA in the complement of condensed metaphase chromosomes was 4 times greater than the normal DNA content.

Many electron microscope observations add support to the concept of the multistranded chromosome. Both thin sections and whole mounts have resolved a 100 A fibril in chromosomes of both animals and plants. Two such "elementary chromosome fibrils" are often associated into 200 A fibrils. The 100 A fibril has been further resolved into two 30 to 40 A units (*22, 61*) and Nebel (*62*) has identified 30 A fibrils in mammalian chromosomes. These lines of evidence are considered in detail in the reviews of Ris (*8*) and Kaufmann, Gay, and McDonald (*7*).

Information on chromosome structure also derives from radiation-induced breakage and reunion. The role played by such studies in correlating DNA replication with chromosome duplication has already been mentioned. Irradiation of late prophase or metaphase chromosomes has yielded an anaphase bridge configuration without an accompanying fragment but has two unstretched side arms attached centrally on the bridge. Crouse (*63, 64*) studied these aberrations in *Lilium* and concluded they provide evidence for breakage and reunion of subchromatids. La Cour and Rutishauser (*65*) have described similar structures in irradiated endosperm, and Brock (*66*) has reported them in nonirradiated endosperm. Wilson, Sparrow, and Pond (*67*) produced these aberrations in *Trillium* and were able to trace the path of the half-chromatids in the bridge: They distinguish between "true" and "pseudo" two-side-arm bridges and predicted that other configurations might be found. Some of the configurations possible with the assumption of rejoining of broken half-ends are shown in text-figure 3: Other patterns of rejoining yield figures which could also be derived from chromatid or chromosome reunion.

DIPLOTENE BIVALENT	ANAPHASE I	DESCRIPTION
		TWO SIDE ARM BRIDGE (TRUE)
		TWO SIDE ARM BRIDGE (PSEUDO)
		Y-CHROMOSOME AND DEFICIENT CHROMATID
		ONE SIDE ARM BRIDGE AND FRAGMENT

TEXT-FIGURE 3.—Anaphase I configurations resulting from some of the patterns of reunion of half-chromatids following a break across a diplotene chromosome. The crossover proximal to the breakage-reunion site ensures a first division figure; second division configurations would occur in the absence of the crossover. [After Wilson, Sparrow, and Pond (*67*).]

Peacock (*68*) induced subchromatid errors in *Vicia* and found similar arrays of configurations in both mitotic and meiotic chromosomes (figs. 10, 11, 12, and 13). He identified both true and pseudo two-side-arm bridges and also observed the other configurations predictable on a subchromatid breakage-reunion hypothesis. The Y-shaped chromosome (fig. 11), which occurs in both mitotic and meiotic cells, is not easily explicable in any other way. Apart from these configurations *per se*, the aberrations often serve to increase the spatial separation of half-chromatids. The identification of two feulgen-positive strands seems certain in the side arm region of the meiotic bridge in figure 10 and in the stretched region of the mitotic bridge in figure 13. These separated strands correspond to the half-chromatids visible in figures 1 and 12. Taylor (*10*) has claimed that half-chromatid errors do not persist through the succeeding interphase and has accommodated half-chromatid breakage and reunion within a single molecule model by proposing a complex system of links. In his scheme "H links" are laid down prior to replication to form a stabilizing framework for DNA replication—breakage and reunion of these axial elements would yield temporary half-chromatid errors. However, Wilson *et al.* (*67*) and Wilson and Sparrow (*69*) have identified aberrations at pollen grain mitosis which were consistent with subchromatid breakage and reunion at meiotic prophase, the time of irradiation. Peacock (*68*) obtained chromatid errors in the second mitosis after irradiation following colchicine suppression of the first anaphase. The frequency of these errors was much lower than might have been predicted from anaphase bridge frequencies but a preponderance of X over U exchanges could account for this discrepancy since pseudo-side-arm bridges would not yield recognizable X_2 errors. Also, a lack of correspondence between the anaphase half-chromatid and the unit of segregation following replication would reduce the X_2 expectation.

Circular DNA Molecules in Chromosomes?

The suggestion of more than one DNA molecule per chromatid, in a lateral sense, seemingly adds complexity to already conceptually difficult genetic problems. The argument presented in the preceding sections has not been developed for simplicity but as the necessary, to the author, conclusion to several lines of data. Some recent observations on oocyte nuclei of Amphibia attest further to the complexity of chromosome structure in higher organisms.

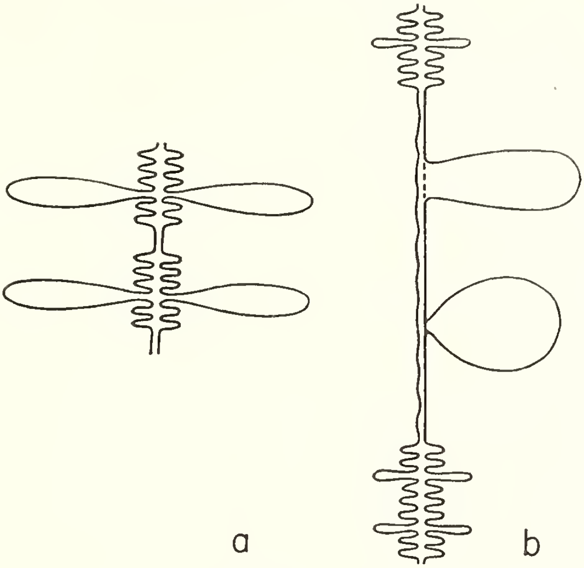
Dr. James Kezer of the University of Oregon has recently begun a study of the oocyte cytology of the Plethodontid salamanders as part of his investigation into the cytoevolution of this group. He has shown the animals to possess lampbrush chromosomes similar to those of the newt discussed by Dr. Miller in the preceding paper, but their most striking feature concerns the nucleoli. Newts and salamanders, rather than having one large nucleolus in the oocyte, have many small nucleoli usually arranged in the peripheral zone of the mature nucleus. In many Plethodontid species Kezer finds that the nucleoli are represented by ring-shaped structures (figs. 14 through 21). The nucleolar rings vary considerably in size and appearance even within oocytes of the one animal; circumference of the rings may be as small as a few microns or as great as 400 microns. Most commonly the rings are 100 to 200 microns in circumference. In some oocytes the rings can be seen to form compact nucleoli. Miller (personal communication) has reported that dispersion of the compact nucleoli of the newt discloses "beaded necklaces"; his finding was completely independent of Kezer's work. Collaborating with Dr. Kezer and Dr. Wimber, I have been involved in an attempt to further characterize these structures. The rings give a positive feulgen reaction indicating that DNA is a component. Enzyme tests have shown this to be the case. Deoxyribonuclease disrupts the circular structure of the rings and eventually reduces them to many small fragments (figs. 18 through 21). Ribonuclease, trypsin, and pepsin all appeared to digest the "beads" but did not disrupt the ring structure. The inference follows that DNA is the component that maintains structural integrity of the rings: The nucleolar rings appear to be circular molecules of DNA with associated RNA-protein complexes forming a coating which is sometimes differentiated into "beadlike" structures. These experiments will be recorded in detail elsewhere. Miller has obtained essentially similar results in the newt.

What of the origin and function of these rings? Some of the Plethodontid species have provided an answer with respect to origin. A particular region of one bivalent appears to be their source point—this region, presumably the nucleolus organizer, bears loops closely resembling the rings which are free in the nucleus (figs. 14 and 15). This locus remains in an extended stage long after the remainder of the chromosome loops have been retracted and can still be recognized in late diplotene. Since there may be as many as 200 to 300 rings per nucleus, it must be assumed that there is some form of extended duplication period for this particular region of the chromosome. Labeling experiments are in progress in an attempt to follow the dynamics of this process and also to check whether there is any replication in the free rings (light microscope observations and size class distribution suggest this as a possibility). Attempts to obtain a system for quantitative analysis of deoxyribonuclease kinetics have thus far been unsatisfactory so the number of strands in the rings remains unknown. Whether they are single- or double-stranded, the mode of origin is of great interest. We have no observations to suggest that the rings are not released as circular structures but it is possible that circularity results from union of ends after detachment from the chromosome. One peculiar feature of this region is that, although the two chromatids are easily identified, the nucleoli loops appear not to arise as paired structures as is the case with other loops in lampbrush chromosomes (*see* text-fig. 4). Apparently genetically identical strands (sister chromatids) are not necessarily involved in the same activity at any given time—this may have some relation to the endoreplication of the rings, but the relationship is certainly not obvious at present.

There is no evidence of the function of the rings, but on the basis of the recently demonstrated role of the nucleolus organizer in the production of ribosomal messenger RNA (*70, 71*), it seems probable that the rings may provide many extra sites for the production of such messengers. Such a function would seem likely in the nucleus of a cell which carries out a vast amount of protein synthesis. Experiments with H^3-uridine have been initiated to check for RNA synthesis on the rings.

CONCLUSION

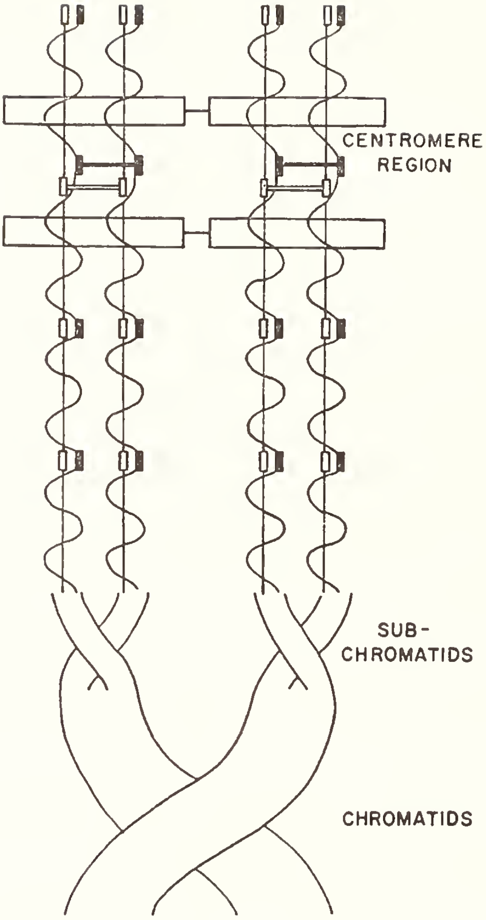
It seems beyond any doubt that the mechanisms of DNA replication and of chromosome duplication (with respect to DNA) are semiconservative. Moreover, there is some evidence that units of opposite polarity exist in the chromosomes just as they do in a DNA double-helix.



Text-figure 4.—(*a*) A diagrammatic representation of a portion of a lampbrush chromosome showing paired loops and chromosomes. (*b*) A comparable diagram for the nucleolar-ring loop region in *Plethodon cinereus.*

However, it does not necessarily follow that the chromosome is, in fact, one DNA molecule. Asynchronous DNA synthesis along the chromosome arm requires that there be discontinuities in the DNA of a chromosome, so that lengthwise there are several units of replication. This is shown diagrammatically in text-figure 5 with the linkers being unspecified. There is some reason to suppose that so-called residual protein may play a role in the linear association of DNA molecules (*72, 73*), but more data is needed on this point. Steffensen (*74*) has demonstrated that divalent metal ions can be of significance in the maintenance of chromosome integrity. But again the facts are insufficient.

The chromosome model in text-figure 5 also shows lateral repeats of the unit DNA piece. Evidence is strong that half-chromatids are a reality: Living and fixed cells show similar subchromatid structures. It is known that these half-chromatids act as units of reunion following chromosome damage induced in late prophase, and it is further known that they contain DNA (on the basis of feulgen staining). The phenomenon of isolabeling in X_2 autoradiographs puts the case forward that the DNA of each half-chromatid consists of at least a double-helix molecule. All of these features are diagramed formally in the chromosome shown in text-figure 5. Electron microscope evidence indicates that further substructures may exist in some chromosomes. If the existence of half-chromatids is accepted, together with the minimal requirement that each consists of a double-helix of DNA, then a system of semiconservative segregation of helices must be imposed in order that this multistranded structure conform to the demonstrated regular semiconservative pattern



TEXT-FIGURE 5.—A diagram depicting a chromosome as demanded by results obtained by X-ray breakage-reunion and autoradiographic experiments.

of chromosome duplication. In the diagram "like" helices of the separate molecules are shown to be connected to each other by undefined bonding in the centromere region. This is meant only to indicate that the segregation requirement has been recognized; a single point of segregation would suffice but it is possible that many such points exist, *e.g.*, at the links between successive DNA molecules. Walen (personal communication) has shown that there is a strictly controlled segregation at chromosome replication in that in X_2 endoreplicated cells of *Potorus* cultures, label segregation is generally of an outside-outside distribution in the two sets of sister chromatids. She invokes the centromeres as a control point in this strict replication pattern.

It is believed that in *Vicia faba* there is a strong case, on the basis of light microscope, X-ray damage, and autoradiographic analyses, for the assumption of at least two DNA pieces per chromatid. There are no data demanding genetic equivalence of these half-chromatids but this would be implied in a multistranded model. A similar condition may well apply in other organisms, but it is unlikely that it should occur to the same degree in all higher organisms. In this regard the electron microscope observations of Miller (this Symposium) and deoxyribonuclease kinetics of Gall (*75*) strongly suggest a single DNA piece construction of the *Triturus* lampbrush chromosome. Genetic considerations have been largely excluded in this treatment but it may be well to note that functional unity need not necessarily imply structural unity. For the most part, satisfactory levels of both genetic and cytological data do not exist for any organisms, but one case may be pertinent here. Novitski, Cohen, and Laird (*76*) have demonstrated that the single chromatid chromosomes of a *Drosophila* sperm can act as double structures in induced breakage and reunion. Correlated with this is cytological evidence of half-chromatids [Cooper (*77*)]; also half-chromatid aberrations have been detected following irradiation of late prophase I chromosomes (Peacock, unpublished).

The demonstration of circular DNA molecules in the nucleolar rings of salamanders and newts raises the possibility that some or all of the component DNA molecules in the chromosomes of higher organisms are structurally circular. Observations on the Plethodontid salamanders indicate that the nucleoli originate from a particular chromosomal locus and are detached as structural rings. The mode of replication of these molecules is not known, but the suggestion must be entertained that DNA in this region of the chromosome may consist of a series of circular molecules. Stahl (*78*) proposed that a chain of circular DNA molecules was consistent with most genetic properties of chromosomes. Circular molecules could be substituted for the linear molecules shown in text-figure 5 without altering any of its characteristcs. Recently Chovnick, Schalet, Kernaghan, and Krauss (*79*) claimed to have eliminated, on genetic grounds, the possibility of circular DNA in the rosy cistron of *Drosophila*, but it is conceivable that along a chromosome a variety of molecular configurations may exist. It may be that some of the molecules in the chromosome are circular for some of the time, say, for replication.

RESUMEN

En este trabajo se discute la replicación de los cromosomas de los organismos superiores desde el punto de vista del ADN, componente del cromosoma.

Asimismo se consideran experimentos con el ADN de los cromosomas de microorganismos en la medida en que los mismos permiten establecer que la replicación de la molécula de ADN, *in vivo* es semiconservativa y secuencial.

Tomando estas propiedades como premisas básicas, los resultados de experimentos autorradiográficos en varias especies de plantas y animales demuestran un tipo de duplicación semiconservativa en los cromosomas de organismos superiores.

La replicación asincrónica a lo largo del cromosoma exige que varias moléculas de ADN intervengan en la integración longitudinal del cromosoma.

Las excepciones autorradiográficas al modelo general de replicación que se han encontrado, han puesto en duda la conclusión de que el cromosoma está constituído

por una "pieza" de ADN que a su vez está integrada por varias moléculas ligadas por sus extremos.

Parece ser que la isomarcación, es decir, la marcación de regiones homólogas de cromátidas hermanas, observada dos divisiones después de aplicado el marcador, es de ocurrencia general.

Para explicar esta isomarcación en cromosomas constituídos por una sola "pieza" de ADN se requeriría que ocurriesen muchos intercambios de cromátidas hermanas sobre regiones muy cortas del cromosoma metafásico: la distribución de los puntos de rotura evidenciados por los intercambios observados no está de acuerdo con esta hipótesis.

La única alternativa posible es que existan más de una "pieza" de ADN dispuestas lateralmente en el cromosoma. Muchas observaciones citológicas de mitades de cromátidas tanto en células vivas como fijadas apoyan esta suposición.

La realidad funcional de mitades de cromátidas es observada en las aberraciones inducidas por rayos X, donde las mitades de cromátidas actúan como unidades de reunión.

Se concluye que, en *Vicia fabia* y talvez en general hay buenas evidencias para aceptar la existencia de más de una "pieza" de ADN por cromosoma.

Se menciona someramente el hallazgo de moléculas de ADN, aparentemente circulares, en los núcleos de Salamandra y Tritón. En las salamandras Plethodon, hay evidencias de que estos "nucleolos en forma de anillo" son reproducidos en sitios específicos de un cromosoma.

Se discute la posibilidad de que las moléculas circulares de ADN sean una característica general de la organización de los cromosomas de los organismos superiores.

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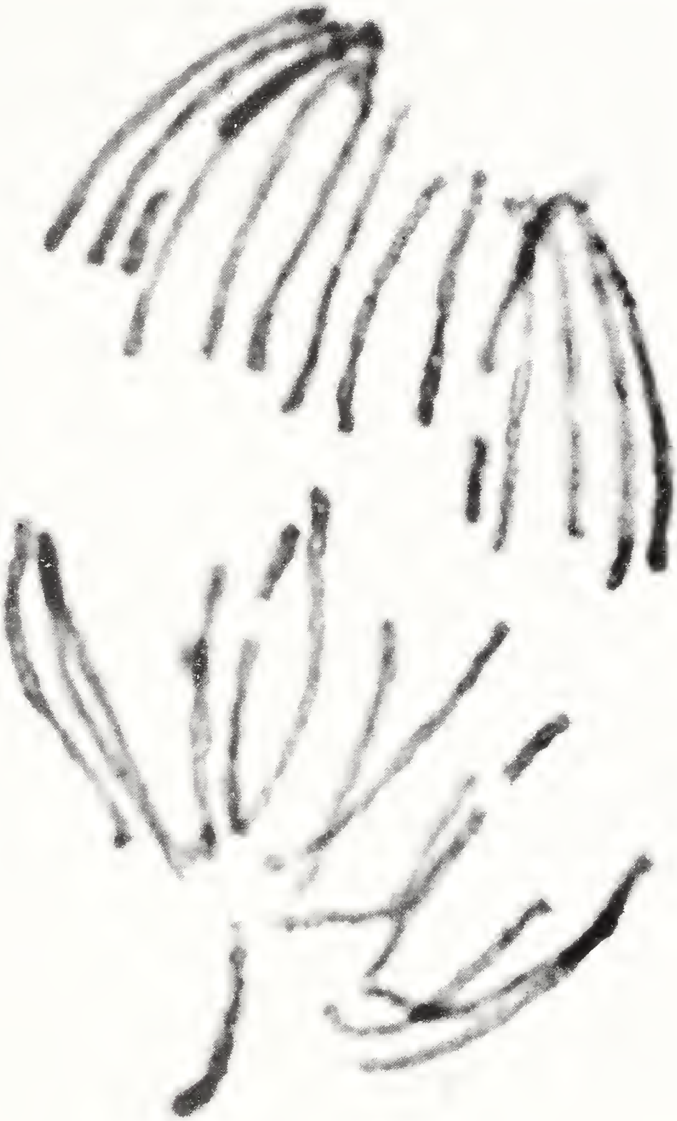

FIGURE 1.—Mitotic anaphase in *Vicia faba* showing 1 pair of metacentric chromosomes (M) and 5 pairs of subtelocentric chromosomes (S) at each pole. Relationally coiled half-chromatids are visible in many of the chromosomes.

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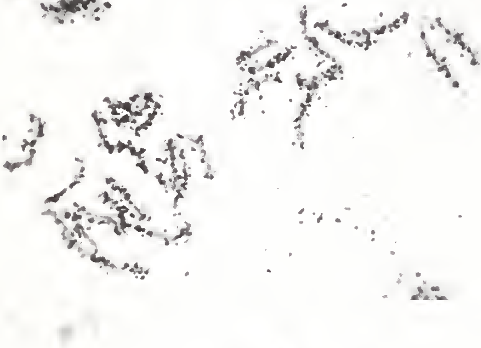

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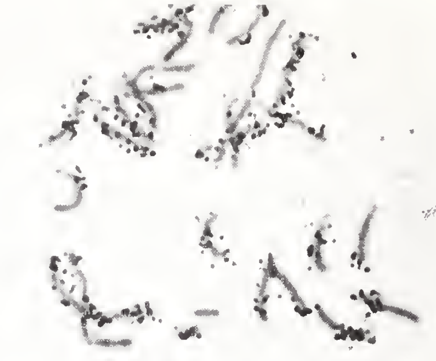

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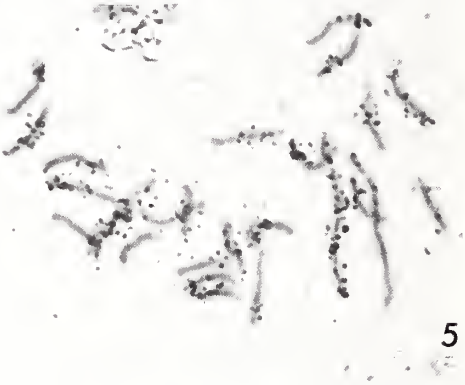

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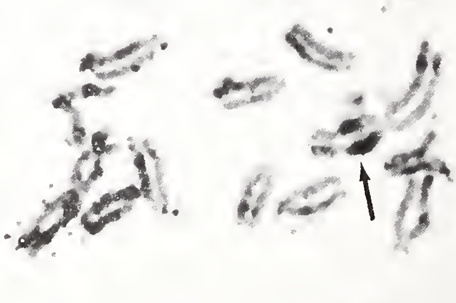

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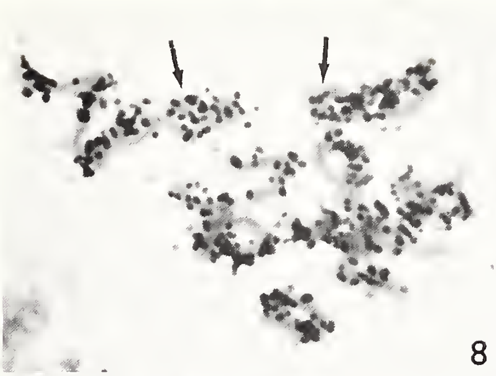
FIGURE 2.—Metaphase of first division after label application showing labeling over sister chromatids of all 12 chromosomes. *Arrow* indicates apparent segregation of label over a trabant.

FIGURE 3.—Colchicine X_1 anaphase showing label over sister chromatids.

FIGURES 4 AND 5.—Colchicine X_2 anaphase showing segregation of label over sister chromatids and also sister chromatid exchanges.

FIGURE 6.—Portion of an X_2 tetraploid metaphase with most chromosomes showing a clear segregation of label. *Arrow* indicates an S chromosome which is isolabeled.

FIGURE 7.—Diploid X_2 metaphase photographed at level of chromosomes. Regions of silver grains show as *dark areas*. *Arrow* indicates an isolabeled segment in an M chromosome.



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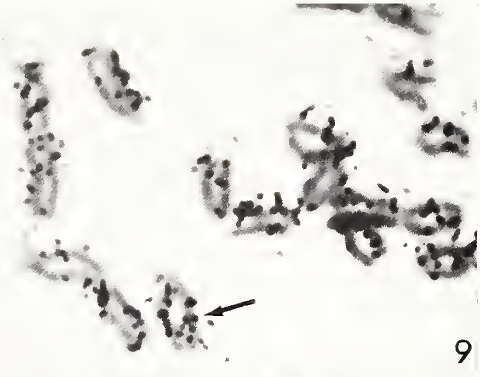

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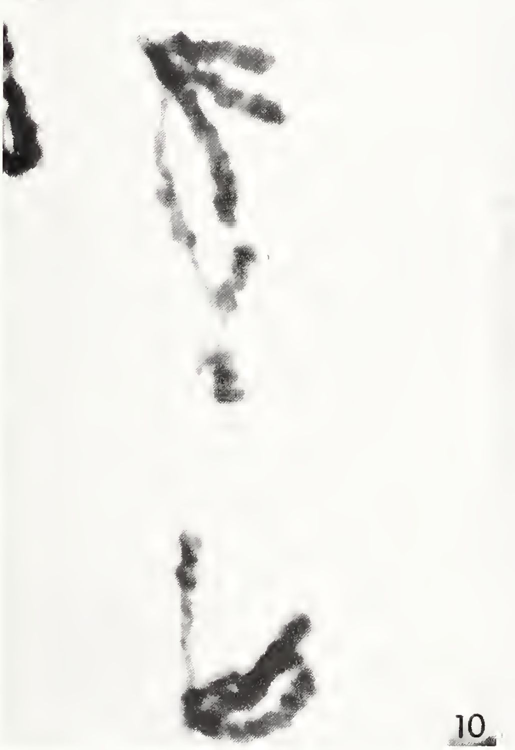

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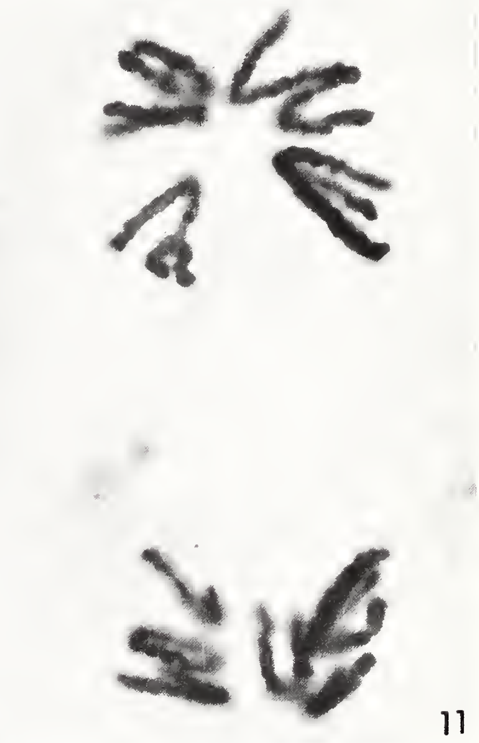

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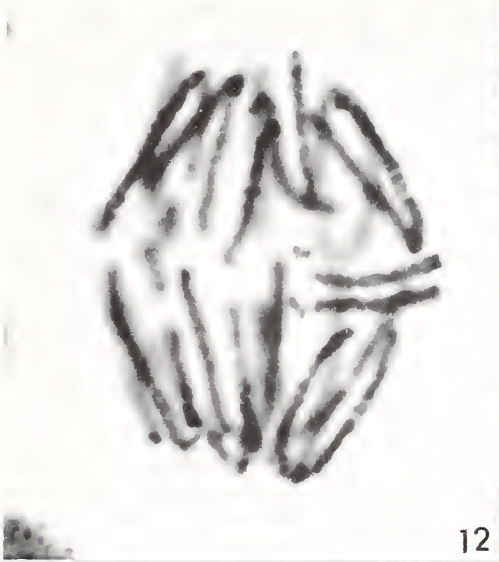
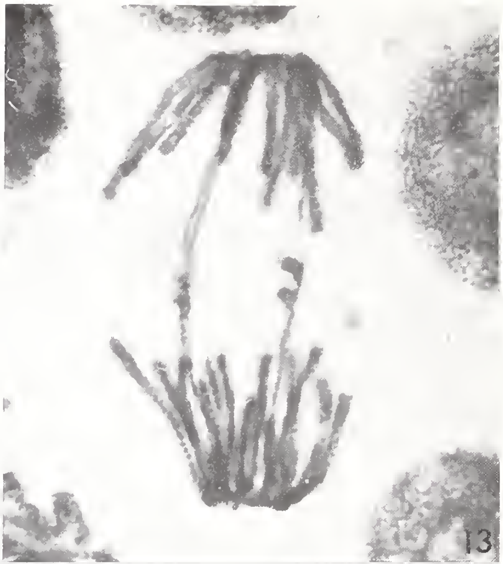
Figure 8.—X_2 diploid metaphase showing mostly label segregation but *arrows* indicate intercalary and terminal segmental isolabeling in the 2 M chromosomes.

Figure 9.—Portion of an X_2 tetraploid metaphase; *arrow* indicates a segmental isolabeling in an S chromosome.

Figure 10.—An M chromosome at anaphase I of meiosis in *Vicia faba* showing a two-side-arm bridge. Bridge appears to have broken on one side of the side arms. Clarity of half-chromatids in the side-arm region permits identification of this configuration as a true two-side-arm bridge, having an acentric half-chromatid piece.

Figure 11.—Anaphase I of meiosis in *Vicia faba* showing a Y-shaped chromatid on an S chromosome at one pole with the corresponding deficiency visible at the other pole.





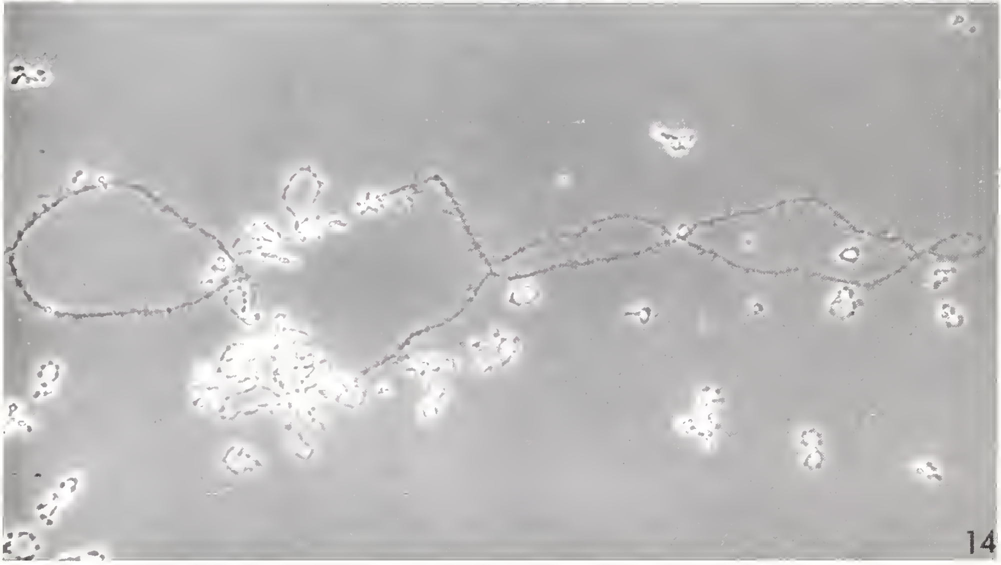
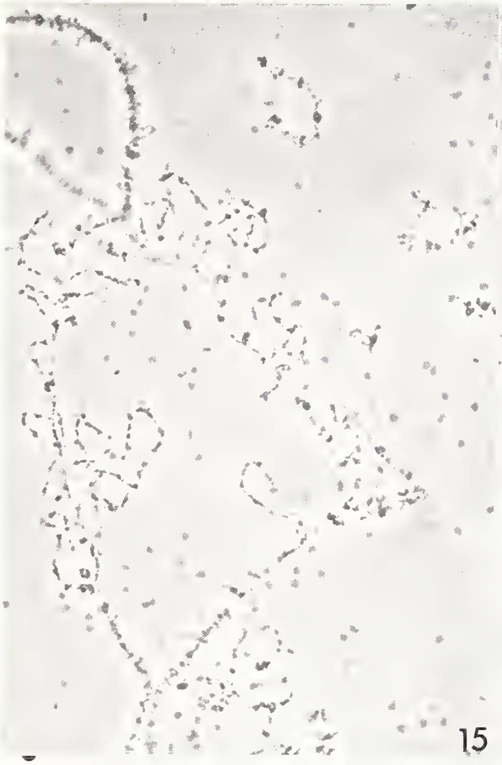


Figure 12.—Mitotic anaphase in *Vicia faba* showing a two-side-arm bridge in an S chromosome with most of the long arms being involved as side arms, the breakpoint being close to the centromeres.

Figure 13.—Mitotic anaphase in *Vicia* where half-chromatids are clearly visible in a stretched region of the two-side-arm bridge. Side arms are small. Another broken bridge is present in the cell.

Figure 14.—A diplotene bivalent from an oocyte of *Plethodon cinereus* showing the "nucleolar ring" locus. A number of nucleoli are also seen free in the nucleoplasm. The bivalent shows 6 chiasmata.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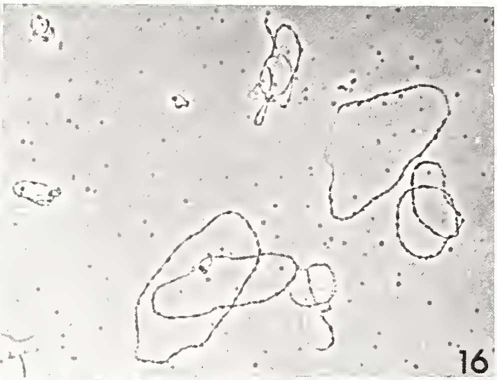
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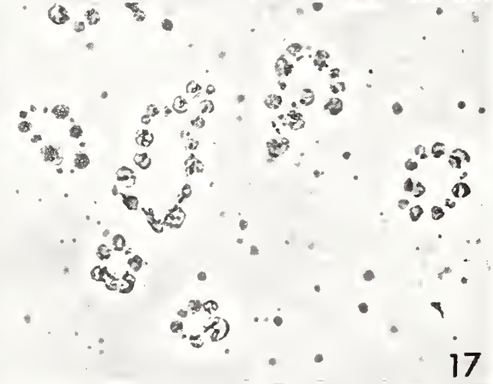
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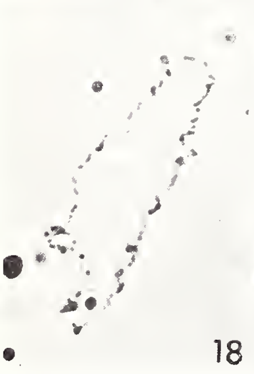
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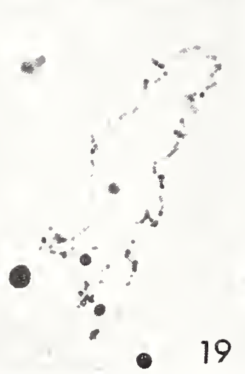
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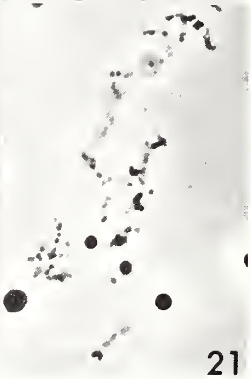
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Figure 15.—Portion of the "nucleolar ring" bivalent of *Plethodon cinereus* showing detail of the region of "nucleolar loops." In many places it can be seen that the "nucleolar ring loops" are not paired.

Figure 16.—Ring nucleoli of *Plethodon vehiculum*, free in the nucleus. Circumference of the larger ones approximates 200 microns.

Figure 17.—Nucleolar rings of *Plethodon cinereus* at the "beaded necklace" stage.

Figures 18 through 21.—A nucleolar ring of *Plethodon vehiculum* showing progressive action of deoxyribonuclease. Photographs cover a period of 30 seconds. First two breaks are visible in figure 19.

DISCUSSION

Miller: Dr. Peacock, in your model does each DNA molecule replicate semiconservatively? If so, does your model not require that at segregation time, when the centromere divides, all of the hydrogen bonds of each DNA molecule must be separated so that the individual polynucleotide chains become disengaged from one another?

Peacock: Yes, each molecule would replicate semiconservatively, but the model does not require simultaneous separation of all the hydrogen bonds of each molecule. The model depicts the centromere region as the site of segregation of the daughter molecules; this segregation could well occur after completion of replication.

Edgar: I would like to expand a point I made earlier, which I think is relevant here, by offering a model of the chromosome, based on Stahl's model, namely, that a chromosome consists of a series of rings linked together. I pointed out that in ring chromosomes of some viruses supercoiling has been found in the structure of the DNA. If we take Cairns' model for the replication of the *Escherichia coli* chromosome, which is also a ring, we see that to replicate the DNA molecule, the whole molecule must unwind. Thus, Cairns invoked a "swivel point," which was a break in the ring, so that the chromosome would not become tangled. However, if the swivel is not 100 percent efficient, replication will then cause the ring to tangle or become "condensed." A subsequent break in the ring will cause it to open. This then provides a molecular mechanism for puffing. Chromomeres would be condensed rings with replication being the driving force for condensation; ring breaks would be the cause of puffing; and ring closure and replication again the cause of condensation.

Moses: I have a question about the frequency of the isolabeling that you observe. According to your explanation of how isolabeling could occur, you could probably calculate the expected frequency of isolabeled chromosomes. Is your observed frequency close to the expected?

Peacock: If whole chromosome isolabeling does in fact result from certain segregation patterns of the component molecules of a chromosome, then its frequency might be predicted; for example, if we assume random segregation on a bineme model, then equal frequencies of label segregation and isolabeling would be expected. However if all isolabeling is actually segmented (being one of the consequences of a sister chromatid exchange event), then we cannot offer a meaningful frequency expectation.

Moses: I think that the question of a single DNA molecule (or chain of molecules) model for the chromosome versus a multistranded model is one that remains open and unsettled for the lack of any really unequivocal evidence. Up to this point, Taylor's experiments and his interpretations of them have been persuasive for a model based on a single chain of DNA molecules, because it constitutes the simplest hypothesis. To fit a more complex multistranded model, one has to invoke a mechanism of the sort suggested by Dr. Peacock for holding like strands of the DNA molecule together. But I do not think there has yet been any unequivocal evidence that leads us to abandon either one or the other of the two concepts. There is no experiment that I know of that cannot be interpreted by alternate explanations.

Peacock: I think this is largely true. Isolabeling is certainly open to an alternate explanation, at least one—that of multiple exchange. We have been attempting to answer this question by increasing the length of the chromosome available for an autoradiograph.

Evans: As chairman, I might make a final comment. My colleague, Mr. Scott, and I are trying to do some experiments using two species of *Allium* which differ in DNA content by about 50 percent—*Allium cepa* with a DNA content of about 70×10^{-12} g and *Allium fistulosum* with a DNA content of about 45×10^{-12} g. These two species both have a diploid chromosome number of 16 and they are genetically extremely similar and hybridize very easily. The *cepa* chromosomes are larger than the *fistulorum* chromosomes and in each cell of the hybrid the size difference is maintained—the hybrid having 8 large and 8 smaller chromosomes. In our experiments we have flash-labeled hybrid root cells and are looking at the patterns of H^3-thymidine labeling at the second, third, and fourth posttreatment mitosis. The slides have only just been developed, so that we have made only a few observations as yet, but I am hoping that we may obtain information on isolabeling and crossover frequencies in these chromosomes, which presumably have a similar genetic make-up but differ in DNA content.

Fine Structure of Meiotic Chromosomes [1,2]

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SUMMARY

Fine details of structure regarding the morphology of meiotic chromosomes at prophase (tripartite groups, synaptinemal complexes) were studied. The most relevant findings are: 1) The medial components of tripartite groups of *Gryllus argentinus* are integrated by a tridimensional fabric of filaments running longitudinally and transversally; the transversal elements interconnect the lateral arms of the tripartite groups; the existence of longitudinal filaments that bend to become transversal suggests that segments of different levels may be interconnected; 2) in *Laplatacris dispar* only transversal filaments are seen; therefore, interconnections are taking place at the same level; 3) groups formed by five alternate lateral and medial elements are found in *G. argentinus* spermatocytes; 4) tripartite groups participate at early prophase in the association of nucleolus and sex chromosome (*G. argentinus*); 5) the elementary component of meiotic chromosomes at prophase and metaphase, as well as their respective kinetochores, is a filament which promedially measures 15 A; high resolution electron micrographs demonstrate that probably two or more than two of these filaments are grouped in a thicker unit about 100 A in diameter.—J Nat Cancer Inst Monogr 18: 133–152, 1965.

THE AIM of the cytologist is to study the structure of cells and to recognize the most intimate units integrating their components. In relation to chromosome structure, the light microscope made it possible to distinguish in the rather large chromosome bodies smaller units, such as the chromatids, the kinetochores, and the chromomeres. With the introduction in the biologic field of the electron microscope, new possiblilities appeared. But the first results were poor, partly due to the crudeness of the preparation techniques and partly to the difficulties inherent in the material itself. In the course of years the impression obtained by many authors is that, in the electron microscope, chromosomes have a monotonous appearance. The difficult problem is to find a way to pass from the microscopic to the submicroscopic dimension. The most important efforts conducted to

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] Research supported by grant GM 08337 from the National Institute of General Medical Sciences, National Institutes of Health, Public Health Service.

relate the already known structure to the intimate details revealed at the level of resolution of the electron microscope were made by Moses (*1–3*), Coleman and Moses (*4*, *5*), Brinkley and Bryan (*6*), and Ris (*7*, *8*). The work of these investigators and of other researchers has contributed largely to the knowledge of the fine structure of the nuclear components.

This paper summarizes three separate findings made in this laboratory on the fine structure of chromosomes at meiosis: 1) the autosomes at meiotic prophase; 2) the elementary components of chromosomes; 3) the sex chromosomes.

MATERIALS AND METHODS

Most of the material examined in this laboratory during the last 8 years was from arthropods of the Orthopterian group. *Gryllus argentinus* (Gryllidae), *Laplatacris dispar* (Acrididae), *Aleua lineata* (Acrididae), and *Blaptica dubia* (Blaptidae) were the species mainly used. Rat and fish spermatocytes or oocytes were also investigated and results on this material were published (*9*). Eventually, one species of Hemiptera, another of Coleoptera and *Acanthopachylus aculeatus* (Opilion) were examined.

The testes were embedded in either *n*-butyl methacrylate or epoxy resins (Epon, Araldite). Sections were made in a Porter-Blum microtome, stained in lead citrate or uranyl acetate, and examined in a Siemens Elmiskop I. Most of the electron micrographs in this paper were Araldite-embedded, uranyl acetate-stained sections.

OBSERVATIONS

Autosomes at Meiotic Prophase

The beginning of meiosis is characterized by the appearance in early spermatocytes of linear structures which, as prophase progresses, become the axis of the chromosome bodies. It is in this period that pairing and concomitant phenomena occur.

The information accumulated in recent years on these linear structures consistently demonstrates the following morphologic facts: Early prophase chromosomes are individualized by the grouping of 3 helically twisted linear condensations of material primarily described as ribbon-like; the 3 ribbons are parallel all along their length (fig. 1). Two less dense spaces (intermediate) separate the medial component from the lateral arms. As currently described, the medial component is less electron-dense than its partners and may even be absent (*4*). Discontinuity along the group length has not been reported nor has confluency or mixing of the opposite lateral components. It is generally accepted, in agreement with Moses (*3*), that each lateral arm of the complex represents a homologous chromosome, and the group the state of pairing (synaptinemal complexes). The concept is based on comparative light and electron microscope studies.

Besides Moses, who first stated the pairing hypothesis, other authors provided additional information: Nebel (*10, 11*), Nebel and Hackett (*12*), Chardard (*13, 14*), and Franchi and Mandl (*15*). The observations by Meyer on *Drosophila* (*16, 17*) are significant because of the interpretation he advanced on the role of the bridges existing between the 2 parallel ribbons.

The material studied in our laboratory some years ago showed a striking prominence of the medial component, which led us to consider this element as the organizer of the chromosome tripartite groups. In *G. argentinus*, for instance, the medial component, instead of appearing as an evanescent structure, was observed to be at the inception of prophase as prominent as its partners (*18*). Transversal continuity through the intermediate spaces was evident at the level of resolution actually obtained (*18*). Many observations at low magnification inclined us to consider each group as forming the core of a single chromosome (*18, 19*). The failure to find the actual act of pairing, that is, the reunion in a group of two single elements, also supported this concept. Cytologists have described the act of pairing as similar to a zipping process, the junction starting from the nuclear membrane.

In the published literature on electron microscopy only one observer reports this phenomenon (*3*). It is a striking fact that our records of studies with the electron microscope, which now include about 5000 pictures, lack a clear example indicating divergence between the two main components of the group.

Moses' hypothesis gained support among other investigators as more research was done and published. Franchi and Mandl (*15*), for instance, demonstrated that single strands occur in the oocyte of the rat at the time leptotene threads are seen in the light microscope, whereas tripartite groups are coincident with the subsequent stages.

Elementary Components of Chromosomes

High resolution electron microscopy applied to the study of synaptinemal complexes resulted in the discovery of certain facts regarding their intimate structure. Some of our results were published in a short note (*20*). In the present paper the description is divided in two parts: The first part analyzes the elements existing in the medial component; in the second, the elementary components of the lateral strands are studied.

The medial component.—To facilitate the description the following terminology has been adopted: Frontal views are those in which the 3 components are seen in full in the same longitudinal plane; lateral views those in which only a face (lateral) of 1 of the 3 elements appear in the longitudinal plane; and cross sections, those in which the 3 elements are seen at their minimum width (estimated to be for most of the species examined not wider than 0.15 μ).

Variations in the structural order of the elements composing the parallel ribbons of the prophase complexes were found in the several species studied. In *G. argentinus* a regularly spaced transversal striation of the medial density is readily observed at low magnification (40,000–100,000), whereas the lateral arms do not show any order. In *L. dispar*, regular order is obvious in the medial components and in the lateral arms. In the pigeon, transversal striation appears in the medial component of the synaptinemal complexes, but it has not the regular arrangement observed in *G. argentinus*. *Acanthopachylus* tripartite groups lack properly identified medial density, but there is a fine fibrillar material interposed between the parallel strands. *B. dubia* prophase chromosomes show a double-lined medial component interconnected with the lateral strands by short projections.

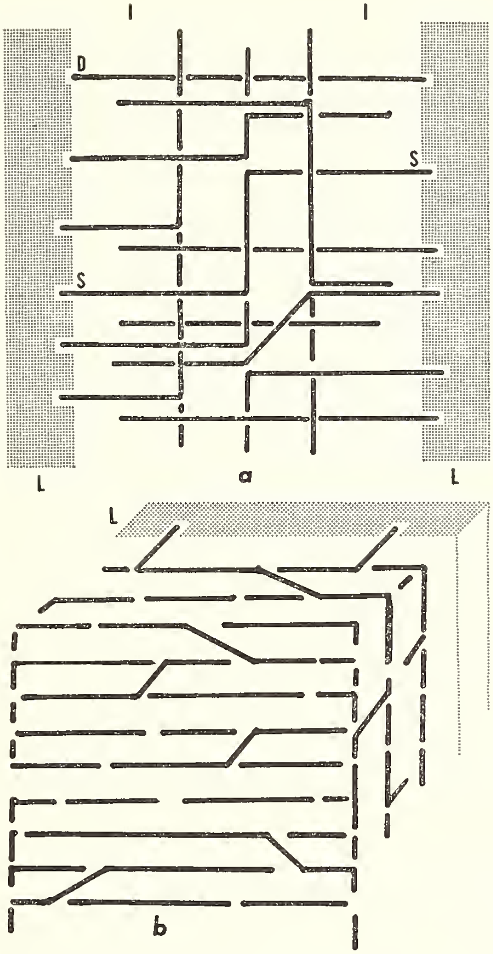
Of the material mentioned above, *G. argentinus* and *L. dispar* deserve a more detailed description.

The medial component of *G. argentinus* is divided into three planes, each formed by longitudinal and transversal filaments (fig. 1). Those running longitudinally look, in frontal views, as if they are the edges of the planes and those that run transversally give to the lateral views of the medial component its striated appearance. Interchange of filaments between planes, as well as change of direction of single filaments, occurs at any place. The two intermediate spaces are crossed by filaments coming from the medial fabric (fig. 2). The crossing filaments may either traverse directly from one of the lateral arms to the other, *i.e.*, interconnecting the arms at the same level (direct crossing) or interrelate different levels of the lateral arms (text-fig. 1) (scalariform crossing). All bridging filaments intermix with the intrinsic element of the strands.

The distance between the planes of the medial density is about 70 A; the separation between stria is about 100 A ($1\mu = 100$ striae). The individual thickness of the filaments composing the planes is about 20 A; the thickness of the striae varies, depending on the viewing angle.

In *L. dispar* spermatocytes, the array of components at the medial place is different from that described above. As can be seen in text-figure 2 and figure 4, there is not a lineal density like in *G. argentinus* or in other species, but a continuous layer of transversal filaments uniting both lateral arms. No intermediate clear spaces are therefore observed. In this species, bridging between both lateral strands seems direct. Interconnection between opposite regions of the lateral densities takes place at the same level. The separating space between bridges is approximately 100 A and the individual morphology of the filaments looks the same as in *G. argentinus*.

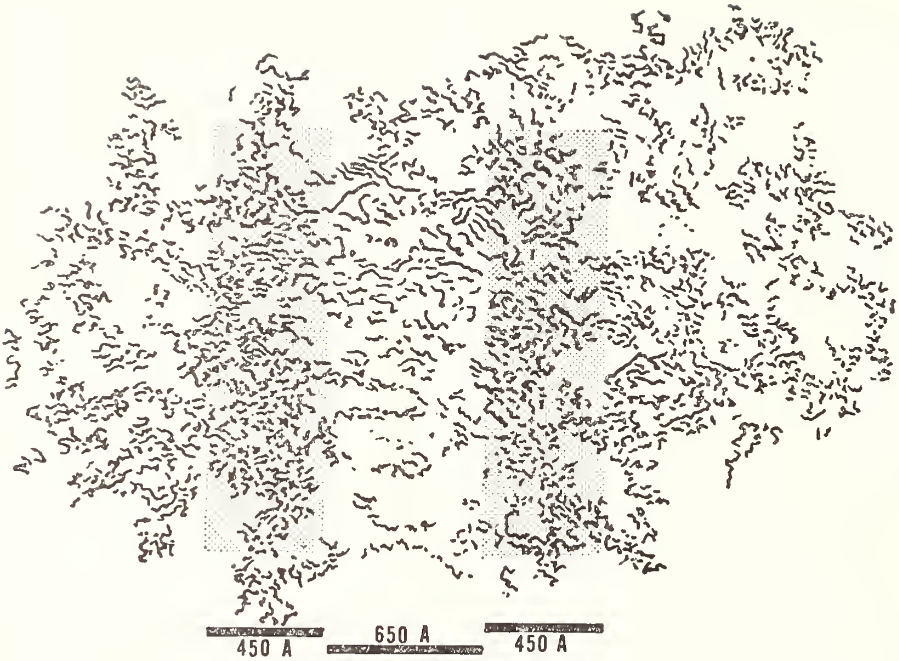
The composite nature of the medial density has been also confirmed in other species (*Coleoptera*) but only *G. argentinus* and *L. dispar* have been closely analyzed. References on the bridging nature of the medial density are found in several publications. In a picture by Gall (*21*) some transversal bridges are seen and Nebel (*22*) represents them in a schematic drawing built on his pictures from the pigeon tripartite group. In a coleopterum, Guenin and Gautier (*23*) described transversal striation along the medial component of the tripartite groups.



Text-figure 1.—The schematic drawings represent the medial fabric in longitudinal (*a*) or in lateral (*b*) views. The trilayered structure of the medial component and the way lateral arms may be interrelated through the crossing filaments (bridges) are represented in both diagrams. Direct and scalariform crossings are lettered D and S, respectively. I: intermediate space; L: lateral arms. Scale: 1 cm = 50 A.

In a recent paper Meyer (*17*) refers to transversal bridging as a probable representative of chiasmata and stresses again that tripartite groups do not occur in *Drosophila* males in which crossing-over does not take place. Because of the increasing richness of detail reported up to now, it is reasonable to assume that the medial component is not a transient structure but an important part of the chromosome axial complex.

The lateral strands.—Most investigators agree that mitotic and meiotic chromosomes are made up of fibrils averaging 100 A thick. The measurements may vary from one case to another, depending on the way the material was prepared and mostly on the level of resolution attained. It is currently accepted, too, that the fibrils found in or extracted from interphase nuclei and those existing in the spermatid nuclei belong to the same category. Theories and representative models on the grouping of active substances within the fibrils or on the grouping of the fibrils themselves within the chromosome have been repeatedly proposed.



TEXT-FIGURE 2.—A direct tracing from a highly enlarged electron micrograph of a tripartite group of *Laplatacris dispar*. The tracing was made to illustrate the filaments traversing the intermediate space, their disposition in the lateral arms (the latter reinforced by two *dotted bands*) and their reunion in fibrils (outside *dotted bands*).

[*See* (*7*, *10*, *22*, *24*, *25*).] Further discussion will concern only the points specifically referred to in this paper.

High resolution electron micrographs from prophase or metaphase chromosomes reveal the intimate structure of the 100 A fibril (figs. 2 and 3). These fibrils do not seem to be independent units, as passage of their elementary components from one fibril to another is frequently observed. In very thin sections (dark) the possibilities of superimposition diminish and the pictures are better analyzed.

The elementary component of fibrils is a 15 to 20 A thick filament coiled along the 100 A space. In fact, it cannot be determined whether more than one filament fills the 100 A space.

The morphology of a single filament is that of an irregularly curled thread showing segments of unequal thickness and density. The length of the filaments is ill defined, due partly to superimposition of elements, partly to irregular binding of the metal stain.

It is estimated that no less than 2 units and perhaps up to 4 may be contained in the 100 A space. A schematic drawing showing the possibilities of array of the filaments according to the information obtained in the electron microscope will be published in another paper on the ultrastructure of metaphase chromosomes (*26*).

It is stressed here that these drawings are shown only to explain the images observed in the electron microscope. No relationship should therefore be seen with models related to the molecular structure of the chromosome-active substances. The following points are noted: 1) The thickness of the filaments is about the transversal dimension of the Watson-Crick double-helix; 2) the separating distance between segments of filaments is about twice the thickness of the filaments; 3) their order in the 100 A space is inconsistent with any regular geometrical structure. A number of factors makes difficult the analysis of the structures and accounts for the lack of constancy in their size or shape: 1) Thickness of sections is twice or more the average size of the threadlike assemblage of filaments; 2) the appearance of the filaments in orthogonal images projected by the electron microscope depends on the orientation of the filaments within the plane of section and these may be of multiple orientation. With thickness of the section decreased, the analysis is made at better advantage, but there is a practical limit for thin sectioning; 3) changes in the morphology of the chromosome components provoked by fixation and pre-embedding procedures may be expected. Some authors (*27*, *28*) have referred to the improving action of divalent cations such as Ca^{++}, but no comparative high resolution studies concerning the 15 to 20 A filaments referred to above have been reported; 4) a closer analysis of the adequate metal stains for high resolution electron microscopy of chromosomes is needed. Most of the studies reported here were made with uranyl acetate. It is observed that metal binding is not homogeneous all along the filaments. This condition makes difficult the estimation of the filaments' length and of their continuity.

Figures 2 and 3 show at magnifications of about 450,000 and 1,250,000, respectively, the array of components in a small part of a tripartite group. The filaments in figure 2 were traced with red wax pencil and the picture was then reprinted. Figure 3 shows an enlarged part of the former reproduced at higher contrast before tracing. The interconnections between the three densities of the complex are obvious in both pictures. Most of the filaments in this segment of chromosome seem to be perpendicularly oriented to the chromosome axis. In the chromosome periphery the filaments are arranged in 100 A fibrils which may be forming loops, as contended by Nebel and Coulon (*22*). Loop-wise array cannot be demonstrated correctly in the thin sections needed for high-resolution work, so another procedure must be devised to investigate this point. Interconnection between 100 A fibrils by means of passage of elementary filaments from one to another makes interpretation of the structure difficult. Higher units such as half-chromatids or chromatids may be formed by groups of interlaced 100 A fibrils.

The individual filaments composing the fibrils are at prophase a little thicker than at metaphase. These data apparently contradict others provided some time ago by De Robertis (*27*, *28*). Small differences in thickness (a few A) may be conditioned to fixation or even to metal staining. It is safer therefore to consider that the filaments keep their diameter from predivision through division.

Two different types of filaments are seen in the lateral arms of *L. dispar* tripartite groups. Filaments about 20 A thick form the inner compact part of the ribbon, whereas 100 A fibrils are in its loose outer part. The 20 A filaments are in longitudinal and transversal order. The filaments separated by 50 A spaces are therefore intercrossed. The 100 A fibrils are irregularly disposed and intermix with the nuclear background (fig. 5).

This paper is devoted mainly to the description of elements. A discussion on their significance is perhaps premature. The evidence accumulated from the research of other authors indicates a number of facts of positive value. Some of these have been mentioned in the preceding pages. The results obtained by Coleman and Moses (*4*), using histochemical methods, point out that the skeleton of the prophase complexes remain unchanged after deoxyribonucleic acid (DNA) removal and also indicate that DNA is located mainly in the microfibrils that are associated with the lateral densities [*cf* (*6*)]. We need more information on the nature of the bridging projections and on the medial density of the complexes. We can obtain this information by combining tracer incorporation with high-resolution electron microscopy.

A recent paper by Woolam and Ford (*29*) on mammalian meiotic chromosome gives a version on the chromatid disposition in the lateral arms of tripartite groups. Nebel and Coulon (*22*) had already mentioned the existence of parallel densities identified as chromatids in each of the lateral arms of the complex. The observations of Woolam and Ford are at variance with Nebel's representation of chromatids. As Nebel sees them, each chromatid would be facing the medial component, but Woolam and Ford estimate that the lateral arms are formed by one inner and outer layer, each representing a chromatid. Doubleness of the lateral arms is sometimes found. In fact we have already pointed out this detail in our first paper on rat spermatocytes (*9*), but the evidence looks insufficient to interpret them as the chromatids observed in light microscopy.

Sex Chromosomes

The study of the spermatogenesis of *G. argentinus* led us to find in the spermatid nuclei a prominent component which is basically integrated by a stack of elements, similar in structure to the intimate components of autosomes at early prophase. Further study permitted us to relate this composite body to the sex chromosome of the species (*30*).

Two facts are prominent in the behavior of the sex chromosome of the cricket: 1) It is associated with the nucleolus at early prophase, and 2) the associated bodies are invaded by more than one tripartite group. In the interphasic lobulated nuclei of spermatogonia the sex chromosome is located in one of the nuclear lobes, whereas the nucleolus is found in a distant place. At this stage the sex chromosome is integrated by a loose chromonema, which in the electron microscope appears denser than the chromatin blocs of the autosomes. After the last spermatogonial division and coincident with the dispersal of the chromatin blocs, the sex chromosome appears as a compact mass attached to the nuclear membrane; it is distinguished from the nucleolus, which is found elsewhere in the nuclear circumference, because of its stronger osmium affinity.

Nucleolar and chromosome conjunction takes place at the beginning of prophase and before tripartite groups appear in the sex chromosome mass. The last elements develop in full within the coupled bodies when the axial complexes of the autosomes develop in the nucleoplasm. They are, like the latter, attached by one extremity to the nuclear membrane. Some of the ribbon-like groups penetrate the nucleolar mass, sometimes flanking one face of the nucleolar material in such a way that one arm of the group is less dense (chromosome material) than the other side (nucleolar material). More than one tripartite group has been observed integrating the complex (fig. 6). Their real number should be determined by spatial reconstruction from serial sections. During prophase, nucleolar and chromosomal components, including tripartite groups, develop into a complex and a prolongation formed by alternate layers of the basic components.

A detailed description has been recently published. The following facts are emphasized here: 1) The tripartite groups penetrating the complex are built at inception like those of autosomes; 2) the intermediate layers found in the composite body are like the medial components of tripartite groups; 3) the multilayered body participates in the kinetics of the cell division but moves poleward in advance of autosomes; 4) the same body is still found in young spermatids (fig. 6). Here again, the typical structure of the intermediate layers indicates their chromosomal nature.

These findings made imperative a wider research in other species. In some cases it was found that at prophase the sex chromosome is homogeneous like metaphase autosomes, as for instance the *L. dispar* (Acrididae), but another Gryllidae species (undetermined) showed a complex body like the sex chromosome of *G. argentinus*. In addition, bodies formed by a conjunction of several tripartite groups were found (*31*) in an *A. aculeatus*, (Opilion), and association of tripartite groups are found in the spermatids of *B. dubia* (*18*).

Except for the observations in *G. argentinus*, the above data presented are fragmentary and do not permit definitive conclusions on the nature of the body concerned. It seems necessary to explore other species for comparative purposes.

RESUMEN

En este trabajo se estudia la estructura fina de los cromosomas meióticos en profase (complejo sinaptinémicos, grupos tripartitos). Los hallazgos más importantes son: 1) los componentes medios de los grupos tripartitos de *Gryllus argentinus* están integrados por una tridemincional de filamentos que corren en sentido longitudinal y

transversal; se observa que el elemento transversal establece conexiones entre los brazos laterales de los grupos tripartitos. La existencia de filamentos longitudinales que se doblan para transformarse en transversales demuestra que segmentos de niveles distintos se encuentran conectados entre sí; 2) en *Laplatacris dispar* sólo se ven filamentos transversales por lo que las conexiones sólo ocurren entre segmentos que se encuentran a un mismo nivel; 3) grupos de cinco elementos alternados laterales y medios fueron hallados en los espermatocitos de *G. argentinus*; 4) se demuestra que los grupos tripartitos en profase temprana participan en la asociación del nucleolo y los cromosomas sexuales (*G. argentinus*); 5) el componente elemental de los cromosomas meióticos en profase y metafase como también de sus kinetocoros respectivos es un filamento que mide como promedio 15 A°; electromicrofotografías de alta resolución demuestran que probablemente dos o más de dos de estos filamentos están agrupados en unidades más gruesas de cerca de 100 A° de diámetro.

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Plate 12

Figure 1.—Longitudinal frontal view of a tripartite group of *Gryllus argentinus*, reproduced at a contrast higher than normal to see better the three planes of filaments composing the medial density. × 390,000

1
0.1 μ

PLATE 13

FIGURE 2.—Small part of a tripartite group of *Gryllus argentinus*. To emphasize elementary filaments, part of them were traced with red wax pencil over a print and then reprinted. *Dotted lines* indicate the medial component and the intermediate spaces at both sides of the latter. Interconnecting bridges and groups of elementary fibrils were traced. M indicates the limit of tracing in this particular field. × 450,000

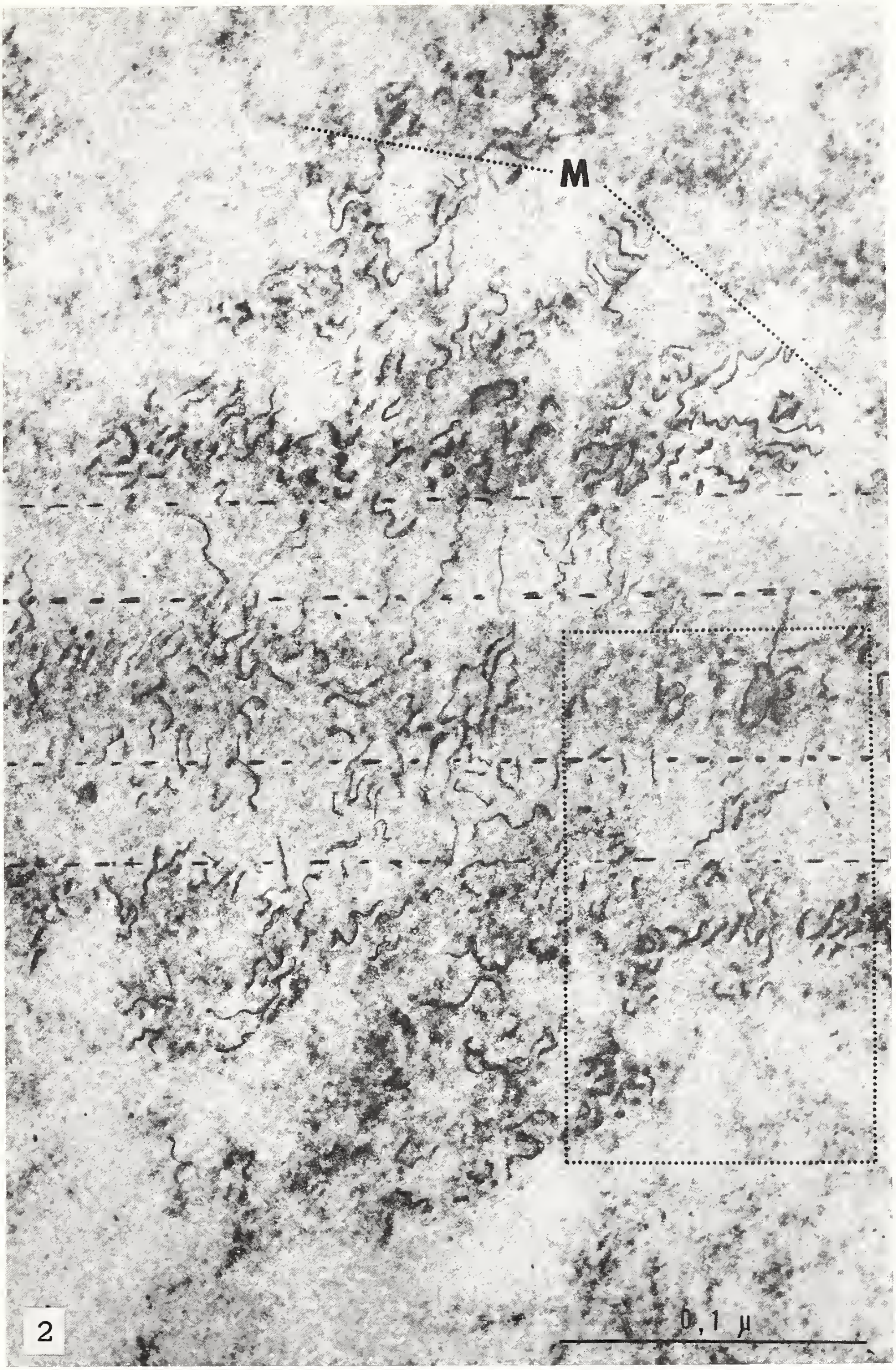
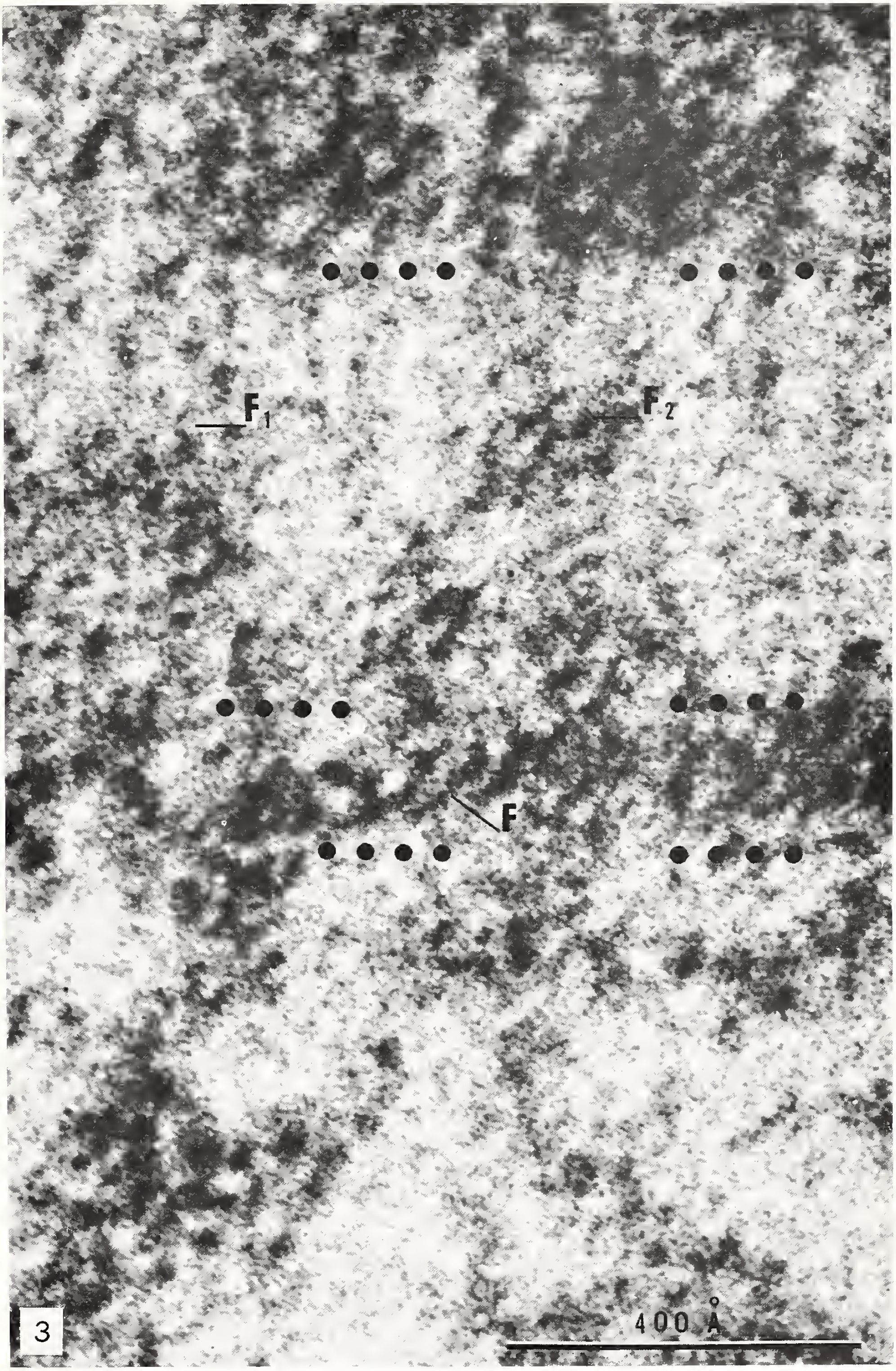
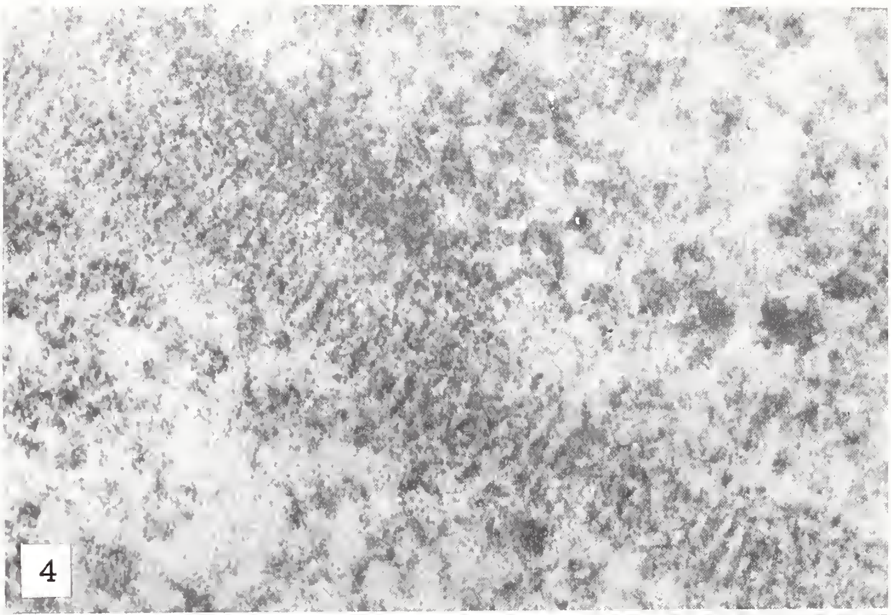

M
2
0,1 μ

Plate 14

Figure 3.—The zone limited by a rectangle in figure 2 is seen here at × 1,250,000. No tracing has been made. Contrast was increased by photographic reproduction. *Dots* indicate at *top* the medial component, and at *center* the compact part of the lateral arm. The group of filaments, F, located between the two central rows of dots deviate toward the left lower angle (periphery of the tripartite group) and those marked F_1 and F_2 interconnect with the compact part of the medial component.


F₁
F₂
F
3
400 Å



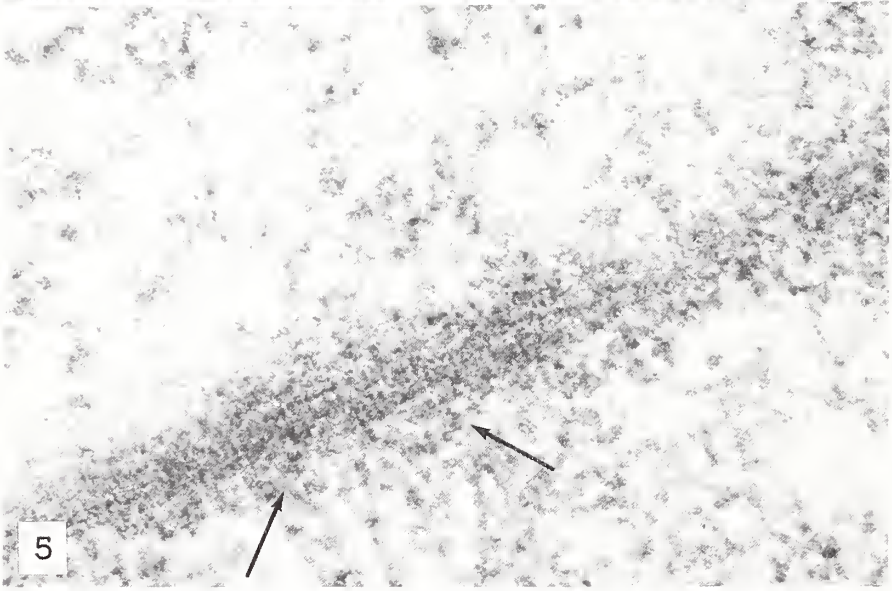


FIGURE 4.—Interconnecting bridges uniting both lateral arms are seen in this tripartite group of a *Laplatacris dispar* spermatocyte. The tripartite group was cut during the course of a helical turn. Part of the medial component appears isolated at *right*. × 210,000

FIGURE 5.—The striated appearance (*arrows*) of the lateral arms is obvious in this lateral view taken from a *L. dispar* spermatocyte I. × 210,000

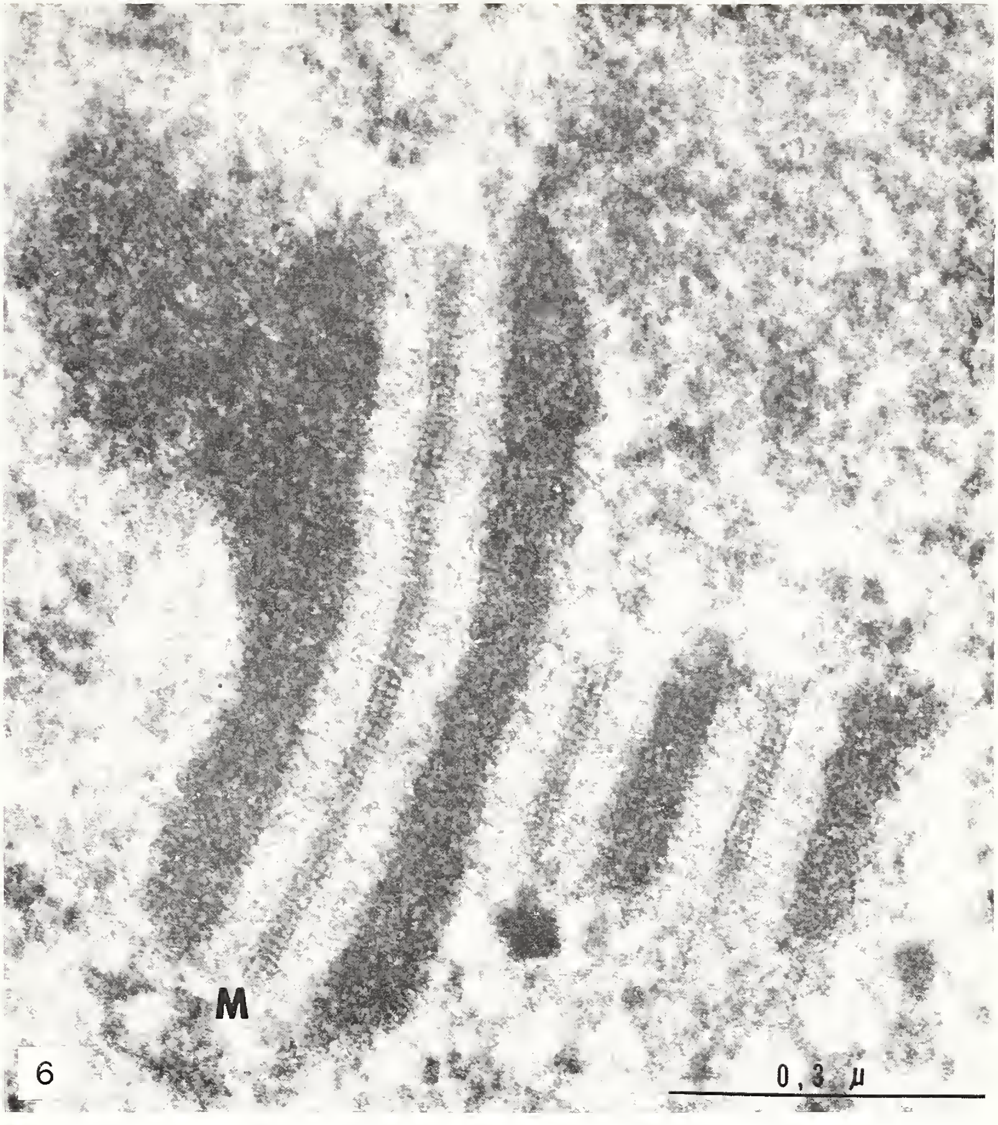


Figure 6.—Multilayered body of a spermatid from *Gryllus argentinus*. The interconnecting bridges between medial components and adjacent layers are clearly seen at high magnification. The medial components, M, show their typical trilayered structure. × 140,000

DISCUSSION

Saez: It is particularly interesting to examine the excellent work done by Dr. Sotelo. I am working with the same species but cytogenetically. The nucleolus has a high density, as it is well known by electron microscopists, but Sotelo says that the sex chromosome is associated with the autosomes in some kind of association that is very curious. Do these sex chromosomes also have a high density? This kind of body is RNA or it is not. If it is a sex chromosome, it is not RNA, and then it should not have a high density. If it is RNA, it is either nucleolus or some kind of matter that is RNA and not nucleolus.

Sotelo: It is not easy to answer such a question. First, I should make it clear that when we started the research on this body we made the Feulgen test to determine which part was nucleolus and which was chromosome. We can affirm that one part is Feulgen negative. I do not know whether this part is all the material of the nucleolus. Sometimes you may find some other nucleolar material in the nucleus. But the point is that at the beginning of prophase, nucleolar material and chromosome material join together. A tripartite group appears in the middle of both these elements, and after these have appeared, they become ordered in a particular way and the body thus formed remains during the meiotic cycle. We have examined other species to look for similar cases. I have found association between tripartite groups in one species of cockroach and in one arachnid species. In other species of *Gryllus* the association of nucleolar material, sex chromosome, and tripartite groups was found to be of the same structural pattern seen in *Gryllus argentinus*. So it is not just a particular and rare case appearing only in some spermatocytes. It is constant in two species and it probably exists in many others. In *Gryllus* it appears in the male and in the female. We can describe it now and nothing more; its significance will be a matter of future investigation.

Lindsley: I think it might be interesting with respect to this discussion to mention some results of Dr. Günther Meyer on these tripartite structures: evidence indicating that they are associated with recombination. In *Drosophila melanogaster* he found the tripartite structure in the females, where crossing-over occurs, and not in males, where there is no crossing-over. Furthermore, he looked at females homozygous for c3G, a gene which completely eliminates meiotic crossing-over, and failed to find any of the tripartite structures. Finally, in females that are heterozygous for complexly rearranged X, second, and third chromosomes, he found a great reduction in the amount of this tripartite structure.

Moses: I agree that Dr. Meyer's observations have very important implications about the functioning of the complex in crossing-over. In line with such attempts to find the complex in cases where crossing-over does not occur, we have examined spermatocyte prophase in a haploid organism, *Stetococcus*. Here there is only one meiotic division; cytologically the prophase is not atypical, except that pairing and chiasma formation are absent. There are two chromosomes in this organism and we have been unable to find the complex in either of them.

Chromosome Breakage and Reunion

On the Chemistry of Chromosome Continuity[1,2]

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SUMMARY

Three different types of experiments performed in our laboratory—experiments on the metabolic requirements for the repair of radiation-induced chromosome breaks, experiments on the types of aberrations induced at various stages of the cell cycle, and experiments on enzyme digestion of isolated mitotic nuclei and chromosomes—have indicated to us that the mitotic chromosomes of *Vicia faba* depend in the main upon nonhistone protein for their linear continuity and are multistranded structures. The results obtained with *Vicia faba* chromosomes, in general, seem to fit those obtained with many other organisms, the one major exception being the results obtained by some workers, but not all, on lampbrush chromosomes of the newt.—Nat Cancer Inst Monogr 18: 155–180, 1965.

CHROMOSOMES of higher organisms consist of nucleic acids and proteins. This much is fact. If, however, we try to be more specific and describe the architectural relations of the chromosome's component molecules, we are soon involved in fancy, for we have no precise knowledge of how the chromosome is constructed either linearly or laterally. That is, we do not know if the linear continuity of the chromosome is maintained by nucleic acid, protein, both individually, or by a nucleoprotein complex, nor do we even know how many strands comprise a chromosome.

Many models have been proposed for chromosome structure and each of these has been based on some, but not all, of the available experimental data. Consequently, these models all differ from one another. Some of these models illustrate the hypothesis that the chromosome has a backbone, that may or may not be protein, from which deoxyribonucleic acid (DNA) extends (Taylor, 1957). Others maintain that the linear continuity of the chromosome is maintained by DNA (Taylor, 1962) or by alternating pieces of DNA and protein (Schwartz, 1958).

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

The models differ more grossly in that some represent a chromosome consisting of several units separated laterally to form a multistranded unit (*see* Kaufmann *et al.*, 1960; Steffensen, 1959, 1961; Ris, 1961), whereas others represent a chromosome that is single stranded.

Over the years we have performed experiments that have given some information about chromosome structure. One type of experiment on the metabolic requirements for the rejoining of radiation-induced breaks can be interpreted as indicating that protein contributes to the linear continuity of the chromosome. A second type of experiment on the type of chromosome aberrations induced by radiation indicates that the chromosome is multistranded. A third type of experiment on enzyme digestion of isolated fixed *Vicia faba* chromosomes has indicated that the mitotic chromosome depends upon protein for its linear continuity and is multistranded.

EVIDENCE FROM EXPERIMENTS ON CHROMOSOME BREAK REJOINING OR REPAIR

X-ray dose fractionation experiments were performed on two-break chromosome aberrations such as dicentrics and rings (Wolff and Luippold, 1955; Wolff, 1960). These aberrations follow two-hit dose kinetics with X rays, that is, they increase approximately as the square of the dose. If the dose is fractionated into two parts such that the breaks from the first dose cannot rejoin or restitute (rejoin in the original configuration) before the breaks from the second dose are introduced, then both groups of breaks will be present concurrently and capable of rejoining with one another to form two-break aberrations. If, however, the time between the doses is sufficient for restitution of breaks to occur, then there will be no possible interaction between the two groups of breaks, and the yield of aberrations will be the sum of the aberrations induced by the first dose plus that induced by the second.

We have performed an extensive series of experiments on G_1 cells in root tips of soaked seed of *Vicia faba*. The two-dose fractions were separated by enough time to allow rejoining of the first group of breaks. We have found that after the first dose was administered (that is, after the first group of breaks was induced in the cells) we could inhibit metabolism in such a way that breaks would remain open instead of restituting. Thus, after the second dose of radiation, all breaks would be in the cell together, be able to rejoin with one another, and the yield of aberrations now would be proportional to the square of the total dose administered. It was found that cellular respiration and the production of adenosine triphosphate (ATP) were necessary for breaks to close. If we inhibited respiration by lowering the temperature, adding cyanide, adding 95 percent carbon monoxide plus 5 percent oxygen in the dark (but not in the light), or by adding dinitrophenol to uncouple ATP synthesis from cellular respiration, then breaks remained open (Wolff and Luippold, 1955). We have similarly found that the addition of exogenous ATP seemed to cause breaks to close faster, as did treatments with manganese chloride, which possibly stimulated the production of endogenous ATP (Wolff, 1960).

All this showed that a source of energy was necessary when bonds were formed during the repair of chromosome breaks. We wondered about the nature of the bonds. Because chromosomes consist of protein and nucleic acids, it seemed worthwhile to see if the synthesis of any or all of these components was involved in the rejoining or repair. We, therefore, performed experiments with the protein synthesis inhibitor chloramphenicol. It had no effect on the yield when given after a dose of radiation to G_1 cells, nor did it have an effect before a dose. When, however, it was administered between two doses, it prevented the breaks from the first dose from rejoining before the second was given. The breaks from the two doses were thus in the cell concurrently and could interact with one another to form aberrations (Wolff, 1960).

Since these experiments were performed in G_1 cells, DNA synthesis was thought not to occur, and this was confirmed in experiments in which we found that tritiated thymidine was not incorporated into those cells at the time the experiments were performed. We also found ribonucleic acid (RNA) was not affected by chloramphenicol treatment in that it did not change the incorporation of $H_3P^{32}O_4$ into the nucleic acids. Chloramphenicol did, however, inhibit protein synthesis in *Vicia*. At the concentrations used (300 μg/ml), chloramphenicol was found to bring about a 30 percent reduction in incorporation of labeled amino acids into the total protein of the cells. We have subsequently checked only nuclear proteins by incubating the cells with tritiated lysine, extracting the nuclei, and making autoradiographs of nuclei uncontaminated with cytoplasmic proteins. Counts of the grains in the autoradiographs showed that there is a 46 percent inhibition of incorporation of labeled amino acids into nuclear protein and, therefore, presumably a 46 percent inhibition of protein synthesis.

Because of the effect of chloramphenicol, and because rejoining can occur throughout the cell cycle (even in stages where DNA synthesis does not normally occur), we postulated that the bonds formed in the repair of chromosome breaks were in protein and not in nucleic acid (Wolff, 1960, 1961). We recognized, however, that the studies on protein inhibition might only have indicated that synthesis of an enzyme, which of course would be a protein, was necessary. The formal possibility still existed that the repair occurred in DNA and required so little DNA synthesis that it could not be picked up in our experiments with tritiated thymidine.

Sandra Bell and I (1964) then performed experiments with 5-fluorodeoxyuridine (FUDR), a compound that inhibits DNA synthesis in bacterial systems. We found it did not prevent breaks in G_1 cells from rejoining. When it was administered between two doses of X rays, the yields were the same as in the controls, that is, simply the sum of the yields produced by the two individual doses. That the compound can inhibit

DNA synthesis in *Vicia* was shown by Bell, in experiments in which she treated actively growing roots that had cells in S. Autoradiographs of FUDR-treated roots showed no incorporation of tritiated deoxycytidine into DNA.

In toto then the observations indicate repair occurs in those portions of the cell cycle in which DNA synthesis does not occur and that inhibitors of DNA synthesis do not prevent repair, whereas inhibitors of protein synthesis do. Thus, we still favor the interpretation that the bonds formed in chromosome rejoining are in protein.

EVIDENCE FROM RADIATION EXPERIMENTS

Radiation studies on the types of induced chromosomal aberrations have long indicated that in late prophase and metaphase an aberration can be induced that ostensibly represents the interaction of half-chromatids (Swanson, 1947; Crouse, 1954; Wilson *et al.*, 1959). This indicates that the chromosome is at least two stranded.

We have carried out studies on the types of aberrations induced in different parts of the cell cycle (Wolff and Luippold, 1964). [Similar studies were performed independently by Evans and Savage (1963).] Actively growing lateral roots of *Vicia faba* were treated with tritiated thymidine for 15 minutes to label those cells in the S portion of interphase. The roots were then irradiated with 150 r of 250 KVP X rays. The cells were sampled every 3 hours and metaphase cells were scored to see the types of aberrations induced and whether or not the cells were labeled. We found that the first cells to reach metaphase came from G_2, *i.e.*, they were unlabeled. These cells had only chromatid aberrations. Later samplings showed labeled cells from S at metaphase. These, too, had only chromatid aberrations. When unlabeled cells from G_1 reached metaphase, they had only chromosome aberrations—the type in which both strands are similarly affected. The presence of this type of aberration indicates that the chromosome was effectively single at the time of X irradiation when the break was produced; and at the time of chromosome replication, the break, too, is replicated.

In an attempt to find just when the chromosome did replicate (act double to radiation) in reference to the time of DNA synthesis, we performed an experiment in which we irradiated first and then administered tritiated thymidine for 15 minutes either immediately or after a delay. It was thought that the delay would allow chromosomes that were broken in G_1 (pre S interphase) to proceed to S where they would be labeled upon duplication. This should give rise to labeled chromosome aberrations. Roots were fixed at successive 3-hour intervals, in an attempt to sample cells that were in different portions of the cell cycle at the time of irradiation and, especially, to ensure that cells were sampled from very early S. Results from such an experiment are presented in table 1, which shows that, if the label was administered immediately after the radiation, no labeled chromosome aberrations appeared even at fixations up to 24 hours. If, however, the application of label was delayed for 1 hour after radiation then an occasional cell that had labeled chromosome aberrations reached metaphase. It was not until the application of label was delayed for 2 hours, however, that a significant number of cells with chromosomes broken in G_1 seemed to proceed to S where they could pick up label to give rise to labeled chromosome, as contrasted to chromatid, aberrations.

TABLE 1.—Labeled aberrations after combined X-ray and H^3-thymidine treatment

Experimental protocol: 150 r, a delay of 0, 1, or 2 hours, and then H^3-thymidine; 200 cells each point, percentages computed from the number of labeled aberrations/total number scored metaphases

Delay (hr)	Time to fix (hr)	Labeled chromatid aberrations (%)	Labeled chromosome aberrations (%)
0	18	33. 5	0
	21	30. 5	0
	24	23. 5	0
1	18	29. 5	0. 5
	21	30. 5	0. 5
	24	24. 5	0. 5
2	18	22. 5	1. 5
	21	18. 5	2. 5
	24	27. 0	2. 0

The simplest interpretation of such results is that, in irradiated material, the chromosome is already acting double to radiation for 2 hours before S. Thus, irradiation of chromosomes before S could give rise to chromatid aberrations that become labeled when the cells move into S. It is as though the chromosome were single 2 hours before S and double from then on.

Such an interpretation, however, presupposes an orderly progression of cells through the cell cycle and presupposes there is no radiation-induced delay of mitosis that would affect the order in which cells arrive at metaphase. Such, however, is not the case: At no time after irradiation are 100 percent of the metaphases labeled as would be expected if there were an orderly progression of cells through the mitotic cycle (*see* Wimber, 1960). Furthermore, some experiments were performed in which autoradiographs were exposed for very long periods so that the number of grains over the nuclei increased to the point where heavily labeled cells could no longer be scored for aberrations because grains obscured the chromosomes. We found that among the cells that could be scored (*i.e.*, were only very lightly labeled) labeled chromosome aberrations did appear even without a delay between irradiation and the addition of tritiated thymidine. The appearance of lightly labeled chromosome aberrations under these conditions indicates that the chromosome is effectively single in very early S before much label becomes incorporated into the chromosomes. Shortly after this, however, the chromosomes, indeed, are effectively double as evidenced by the fact that those cells that are heavily labeled, and so observable with short exposures, always have only chromatid aberrations in them. We could not distinguish whether the lightly labeled cells with chromosome aberrations in the long exposure experiments had been in S at the time they were broken, or had been in G_1, and later, when the cells proceed into S, picked up a small amount of label that could not be washed from the cells. The resolution of the method, therefore, is only good enough to allow the statement that the chromosome acts double to radiation either before S or in very early S before much DNA has been duplicated. In either case, we are left with an indication that the chromosome is at least two stranded in G_1.

E. H. Y. Chu (manuscript in preparation) has carried out studies on chromatid aberrations induced by ultraviolet (UV) irradiation of mammalian cells in tissue culture. He has found that the action spectrum for the efficiency of various wavelengths of UV in inducing aberrations peaks around 2650 A, indicating that energy absorbed in DNA can induce breakage. If, however, chromosome breaks were induced only in DNA, then we might expect the action spectrum to closely resemble the UV absorption spectrum for DNA and to drop sharply at 2800 A. His action spectrum did not drop as sharply as a DNA absorption spectrum at this wavelength. If his data are taken at face value, they indicate that the energy that breaks chromosomes can be absorbed in both DNA and protein, perhaps even in a nucleoprotein complex. Chu has also found that irradiation with 2650 A induces mainly chromatid breaks, whereas irradiation at 2800 A causes a great deal of what he calls chromosome demolition. If he irradiates at 2650 A, a wavelength that is efficiently absorbed by DNA, and subsequently treats with puromycin, a protein synthesis inhibitor, then he obtains demolished chromosomes much like those found when he irradiates at 2800 A. This possibly implicates effects in protein even when irradiations are carried out at 2650 A.

STUDIES ON ENZYME DIGESTION OF CHROMOSOMES

Trosko, who has been working in my laboratory, has recently treated isolated formalin-fixed chromosomes of *Vicia faba* with various enzymes (Trosko and Wolff, 1965). The enzymes were trypsin (0.1 mg trypsin/ml of 0.01 M phosphate buffer, *p*H 7.2), DNase (1.0 mg DNase/ml of 0.003 M magnesium sulfate in 0.1 M phosphate buffer, *p*H 6.8), RNase (1.0 mg RNase/ml of 0.01 M phosphate buffer at *p*H 7.0), or pepsin (1 mg pepsin/ml of 0.1 N HCl at *p*H 1.3). One drop of isolated nuclei and chromosomes in phosphate buffer was mixed with 3 drops of enzyme solution on a microslide which was then incubated in a moist chamber at 35° C for 1 hour.

At the end of this time the preparations were air-dried and stained for DNA by the Feulgen procedure, for RNA with azure B, or for histones by the alkaline fast green method of Alfert and Geschwind (1953). The latter was modified in that the material was hydrolized in hot 5 percent trichloroacetic (TCA) acid for 1 hour instead of only 15 minutes.

The isolation procedure gave preparations of nuclei that were relatively free from cytoplasmic contamination (fig. 1). In figure 2 we see a portion of the preparation containing both free nuclei and clumped metaphase chromosomes that are caught in cytoplasm.

A summary of the effects of various enzyme treatments is presented in table 2. It may be seen that pepsin, DNase, and RNase do not appreciably affect the morphology of the nuclei and chromosomes. This is true even though pepsin removes histones, *i.e.*, the preparations became alkaline fast green negative. DNase and RNase removed DNA and RNA, respectively, as shown by Feulgen negativity or azure B negativity of the slides.

TABLE 2.—Effect of various enzymes on isolated nuclei and chromosomes of *Vicia faba*

Treatment	Alkaline fast green	Feulgen	Azure B[†]	Appearance of treated nuclei and chromosomes
Trypsin (short hydrolysis)	−[*]	+	+	Chromosomes elongated and split into subchromatid strands; nuclear matrix removed, leaving reticular skeleton
Trypsin (long hydrolysis)	−	+	+	Structural integrity completely disrupted
DNase	+	−	+	Chromosomes swollen, but seemingly intact
RNase	+	+	−	No apparent change in structure
Pepsin	−	+		Chromosomes and nuclei slightly shrunken

[*] Alkaline fast green-stained, trypsin-treated chromosomes exhibit no demonstrable stain, yet under phase optics, nuclear skeletons and chromosomes are intact (DNA is removed by the hot trichloroacetic acid hydrolysis used in this staining procedure); − = negative staining reaction; + = positive staining reaction.

[†] Azure B is a metachromatic stain that stains DNA green and RNA purple; a + in this column indicates purple staining.

Trypsin hydrolysis for 1 hour, however, had a considerable effect on chromosome and nuclear morphology in addition to rendering the preparations alkaline fast green negative. If interphase nuclei and chromosomes were watched under the microscope while they were being treated with trypsin, the nuclei could be seen to swell and become progressively less refractile so that eventually they could not be seen with the light microscope. The enzymes seem to digest away some of the material in these nuclei. If after 1 hour of treatment and air-drying the nuclei were observed with phase-contrast microscopy, they were found to have expanded to about 3 times their original diameter (fig. 3). They clearly showed a network of fibers within the nucleus.

Similarly, clumped metaphase chromosomes were observed to separate from one another and stretch by a factor of 3. Figure 4 presents a normal metaphase of *Vicia faba*. After air-drying, the stretched and partially digested metaphase chromosomes are found to be multistranded. Each chromatid consists of two strands, or half-chromatids, that are relationally coiled about one another. The enzyme treatment evidently reduces the number of coils, and in many instances we have found that the two half-chromatids essentially lie side by side (figs. 5 and 6). In certain favorable cases (fig. 6), we can see that each half-chromatid is in itself composed of two interwound strands, indicating that at the light-microscopic level the chromosome is at least quadripartite. (*See also* Gimenez-Martin *et al.*, 1963.) We have occasionally obtained preparations in which the uncoiling has gone on to the extent to which we can find the chromatids bifurcating. We have been able to follow some of the bifurcations to the point at which we can see the four parts of the individual chromatid (fig. 7).

Although the expanded nuclei (nuclear skeletons) and elongated chromosomes left after trypsin digestion are fast green negative, indicating that most of their histones have been removed, they are still Feulgen positive, indicating that DNA is still present. Some DNA has been released from the nuclei and chromosomes, however, as evidenced by the fact that in the Feulgen preparations the general background is now light pink. It should be pointed out that although some DNA is left behind in the skeletons and chromosomes, DNA does not appear to be the architectural component on which the linear continuity of the chromosome depends. For instance, in those trypsin-treated preparations that were stained with fast green, all DNA was removed by the 1-hour hydrolysis in hot 5 percent TCA (90° C). The preparations were Feulgen negative, azure B negative, and fast green negative, yet the skeletons and chromosomes were unchanged and could still be seen with phase-contrast microscopy.

Since pepsin, which also leads to alkaline fast green negative preparations, does not cause stretching and splitting of nuclei and chromosomes, we do not believe that the trypsin effect is caused by a simple removal of histones.

Trosko and I interpret these experiments to indicate that nonhistone protein contributes to the linear continuity of these mitotic chromosomes and that the chromosome is a multistranded structure embedded in a matrix of nonhistone protein.

The regular appearance of multistranded chromatids at metaphase confirms observations that have been made with the light microscope in the past. Kaufmann (1931) and Nebel (1932, 1937) both observed at least two and on occasion four strands in a chromatid. Similarly, Manton (1945) found the chromatids of the fern *Todea* to be bipartite, and Gimenez-Martin *et al.* (1963) found the chromatids of Sicilla to consist of two interwoven strands. The extensive uncoiling that occurs after trypsin treatment in Trosko's experiments, however, can lead to a total unwinding of chromatid strands so that the multistranded nature of the chromosomes is particularly clear. Taylor (1962) has proposed a model of the chromosome (based on Freese's 1958 model) that could explain the appearance of half-chromatids from what is basically a single-stranded chromosome. His model consists of two chains of links that are parallel. These are interconnected by a runglike arrangement of DNA molecules. If, under some conditions, only the two parallel chains of links were observable, the chromatid would have the appearance of being bipartite. The appearance of four strands within a chromosome, however, and the appearance of doubly bifurcating chromatids do not seem to be consistent with such a model.

The appearance of multistranded chromatids not only is consistent with previous observations of chromosomes but also fits the results of radiation experiments in which half-chromatid aberrations were induced at metaphase and chromatid aberrations at the end of G_1 or very beginning of S. These results do not, however, fit the observations of Gall (1963) on the kinetics of DNase-induced chromosome breaks of lampbrush chromosomes. Gall has found that within loops DNase breaks chromosomes with approximately two-hit kinetics. This is interpreted as indicating that, in the loop, the chromosome consists of but a single molecule of double-stranded DNA, *i.e.*, the loop is single stranded. In the interloop regions of the lampbrush chromosome where the strands from two loops come together, breaks are produced with about four-hit kinetics. In addition, the results of our enzyme digestions do not parallel the results obtained by MacGregor and Callan (1962) on lampbrush chromosomes in which they found that DNase, but not proteases, can break the chromosomes.

We are left to wonder if meiotic lampbrush chromosomes differ from the usual mitotic chromosomes observed in plants and animals. If we make a balance sheet, it seems that most of the pertinent observations with the light microscope (Kaufmann, 1931; Nebel, 1932, 1937; Swanson, 1947; Manton, 1945; Wilson *et al.*, 1959; Ris, 1961; Gimenez-Martin *et al.*, 1963; Trosko and Wolff, 1965) indicate that the mitotic chromosome is multistranded, whereas experiments on lampbrush chromosones do not necessarily fit this pattern (MacGregor and Callan, 1962; Gall 1963). It should be pointed out in spite of the experiments of Gall, and those of MacGregor and Callan, work by Ris (1961) on the same chromosomes has led to the interpretation that lampbrush chromosomes, too, are multistranded.

The semiconservative distribution of DNA into daughter chromatids as first discovered by Taylor *et al.* (1957) is simple to visualize in terms of a single-stranded chromatid, *i.e.*, a chromatid consisting of but one double-stranded DNA molecule. Peacock (1963) has recently repeated Taylor's autoradiographic studies and found that, although the majority of the chromosomal label is distributed in the fashion discovered by Taylor, very frequently two chromatids of the second division are labeled at the same level, *i.e.*, are isolabeled. This led him to believe that the chromosomes consist of at least two strands rather than one (*see*, however, Wolff, 1965, for another possible interpretation of Peacock's results).

Although it is easy to visualize Taylor's results in terms of a single-stranded chromosome, it is possible to make schemes whereby a chromosome consisting of more than one DNA double helix distributes its label so that both chromatids will be labeled at the first division and only one chromatid would be labeled at any successive division. Such schemes have been proposed by Schwartz (1958), Steffensen (1961), Cavalieri and Rosenberg (1961), and Peacock (1963).

In figure 8 we present a model that is similar to one proposed by Uhl (1965) to explain crossing-over. This model can account for the segregation of label among daughter chromatids when the chromatid is multistranded. In the model several strands of DNA run side-by-side longitudinally between links. Upon replication, each strand of the double-stranded DNA helix is attached to opposite sides of the plane of splitting of the link. Following one round of replication in label, each strand of the multistranded chromatid will now be labeled and both chromatids will appear to be labeled. If these chromatids now replicate in the absence of label, all the labeled DNA would be in one chromatid, whereas the sister chromatid would be completely unlabeled.

We do not intend this model to indicate that we believe the chromosome is constructed in this fashion. The model in figure 8 obviously does not include all of the features necessary from our data, *e.g.*, DNA not seeming necessary for the linear continuity, and the presence of a matrix. It only demonstrates that in the absence of any critical knowledge of how a chromosome is constructed, it is easy to conceive of an arrangement of the DNA in multistranded chromosomes that could give the results first observed by Taylor and his co-workers, results that must be considered in any formulation of chromosome structure.

Unfortunately, a multistranded chromosome makes it difficult for us to visualize many genetic phenomena, such as mutagenesis and crossing-over. The fact that it is more difficult to think of these processes in terms of multistranded chromosomes than it is in single-stranded ones, however, does not constitute evidence that the chromosome is single stranded.

RESUMEN

En conclusión diré que tres tipos diferentes de experimentos hechos en nuestro laboratorio, experimentos sobre los requerimientos metabólicos relacionados con la reparación de roturas cromosómicas inducidas por las radiaciones, experimentos sobre los tipos de aberraciones inducidos en varios estadios del ciclo celular y experimentos sobre la digestión enzimática de nucleos mitóticos y cromosomas nos han indicado que la continuidad linear del cromosoma mitótico de *Vicia faba* depende principalmente de una proteína no histona y que poseen una estructura formada por varias fibras.

Los resultados obtenidos con los cromosomas de *Vicia faba* parecen estar de acuerdo en general con aquellos que se han obtenido en muchos otros organismos.

La excepción más importante radica en los resultados obtenidos por algunos autores, no todos, trabajando con los cromosomas plumulados de Salamandra.

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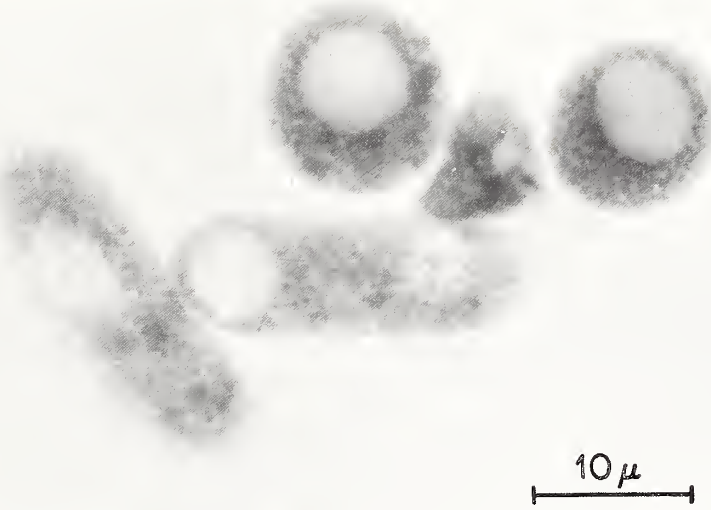
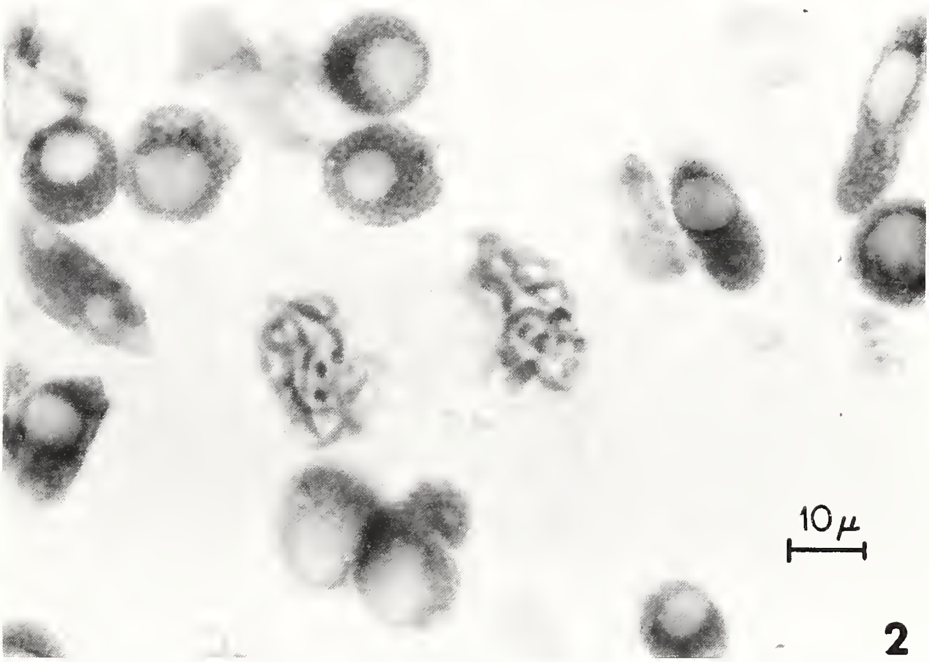
PLATES



1



2

Figure 1.—Feulgen-stained isolated *Vicia faba* nuclei.

Figure 2.—Feulgen-stained isolated *Vicia faba* nuclei and chromosomes.

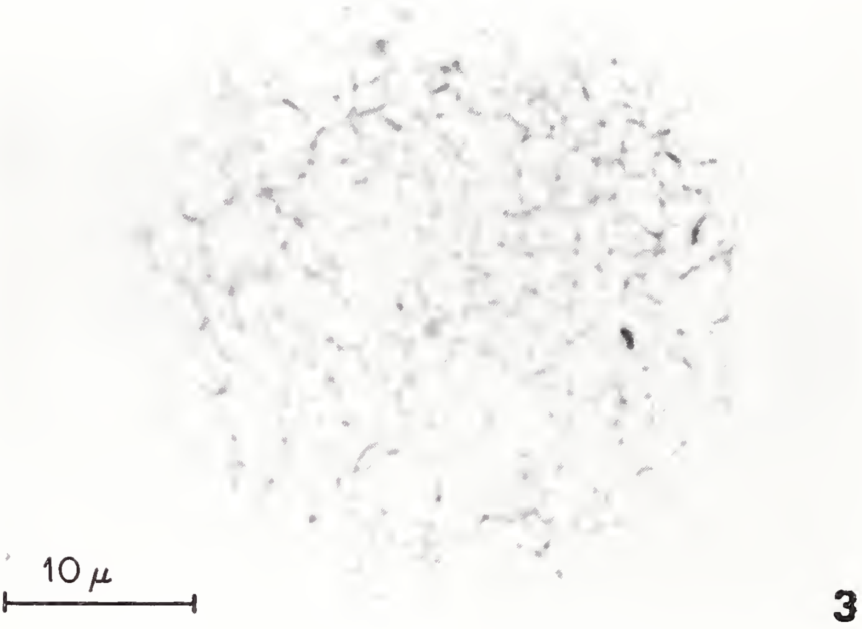


Figure 3.—Feulgen-stained, mildly trypsin-treated isolated nucleus showing skeleton.

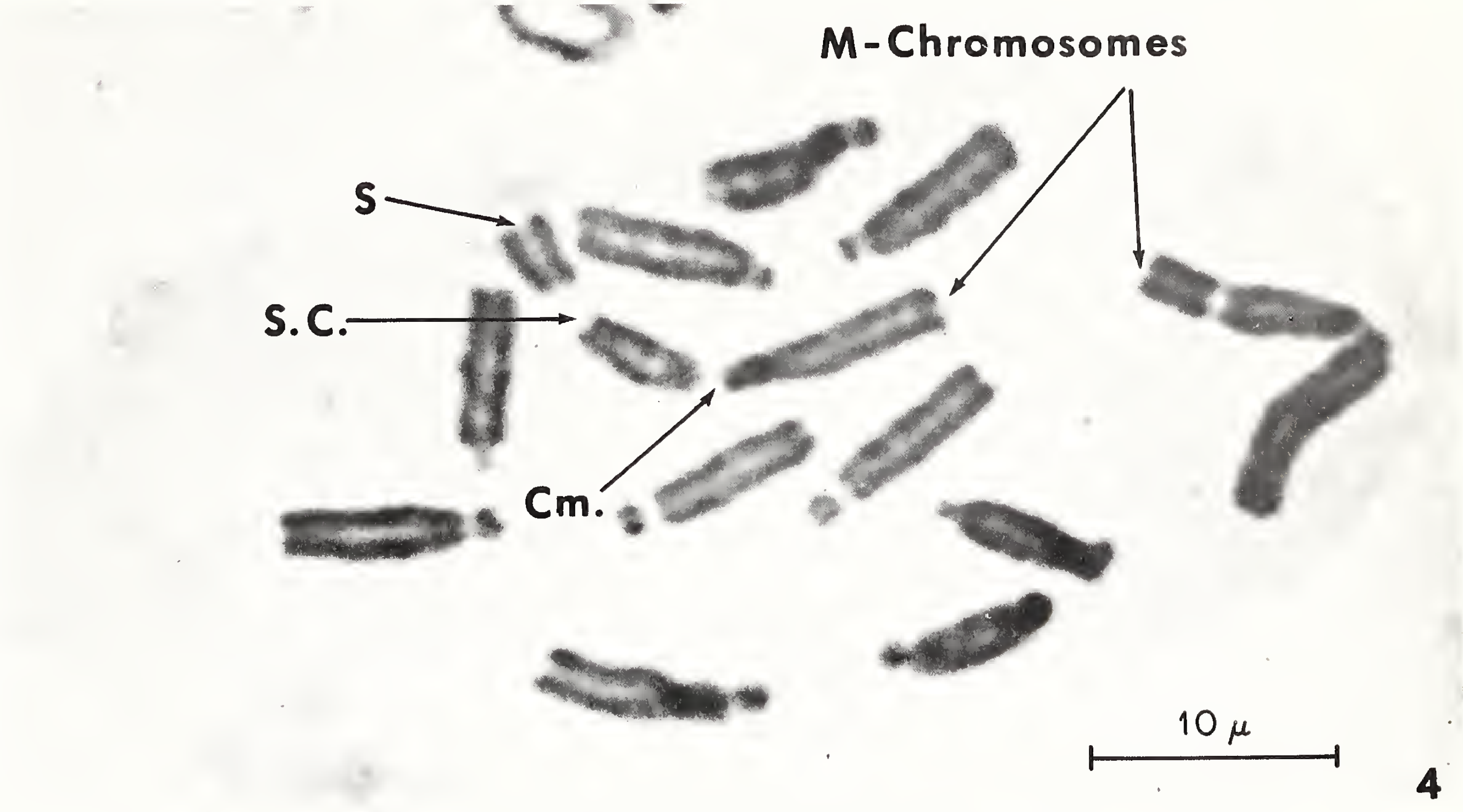


FIGURE 4.—*Normal Vicia faba* chromosome complement at metaphase.

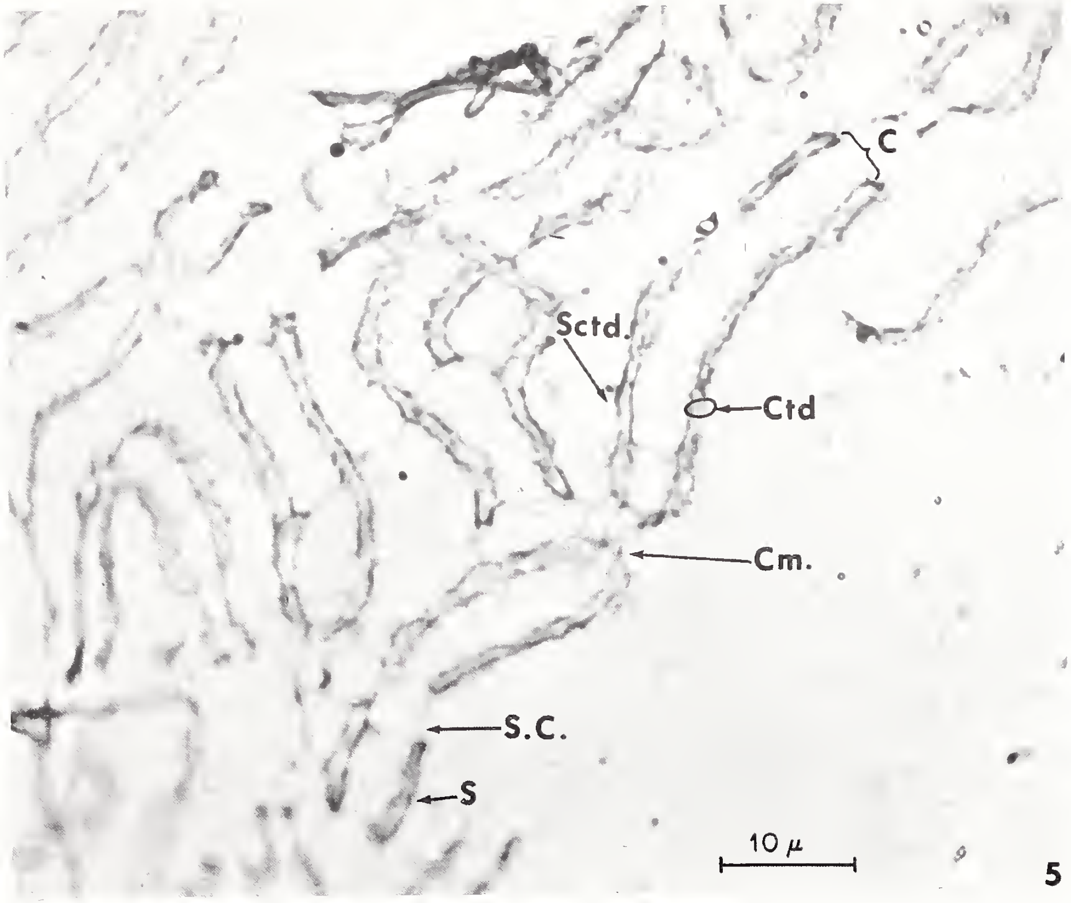


FIGURE 5.—Feulgen-stained, trypsin-treated isolated metaphase chromosomes. The M chromosome (C) is seen with each of its chromatids (Ctd) split into 2 subchromatids (Sctd). The satellite (S), secondary constriction (S.C.), and centromere (Cm.) are also visible.

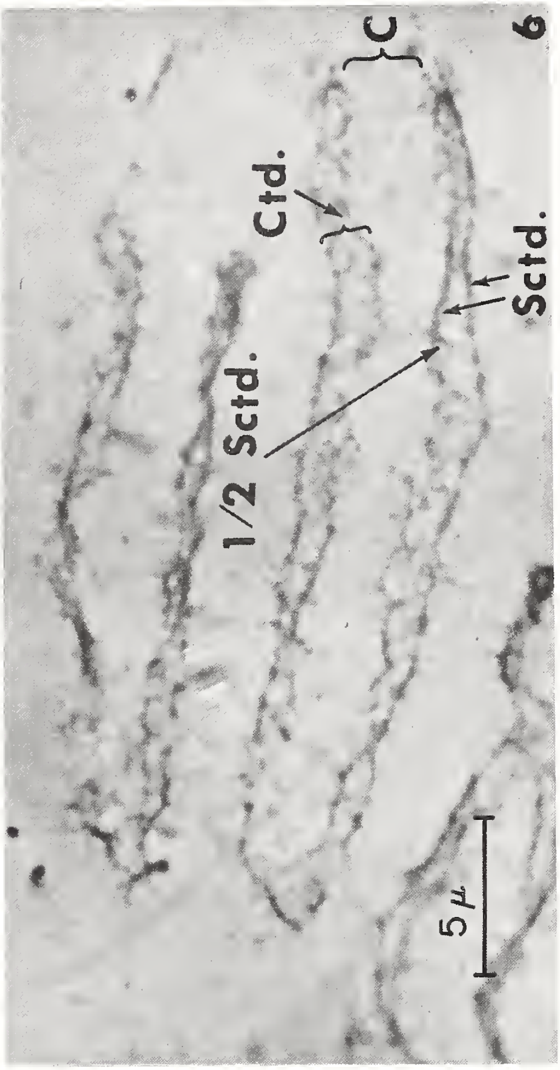


FIGURE 6.—Feulgen-stained isolated metaphase chromosomes.

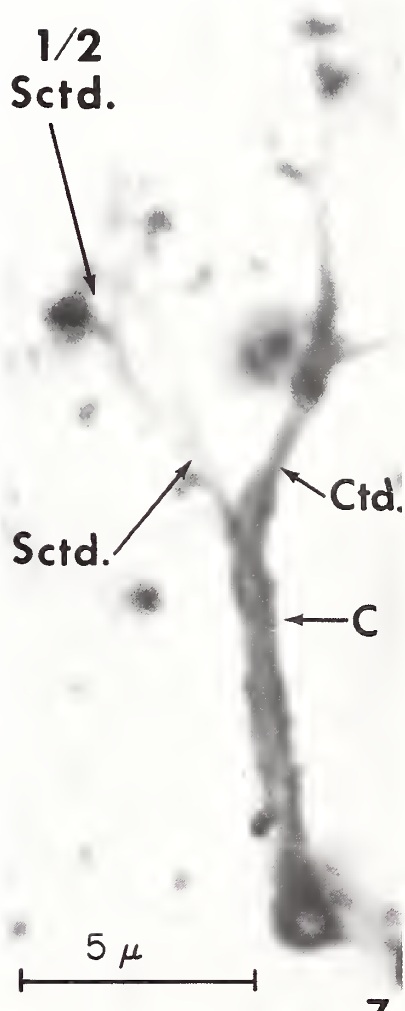


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Figure 7.—Feulgen-stained, mildly trypsin-treated metaphase chromosome showing 1 chromatid separated into half-chromatids, with each of these bifurcating into quarter-chromatids.

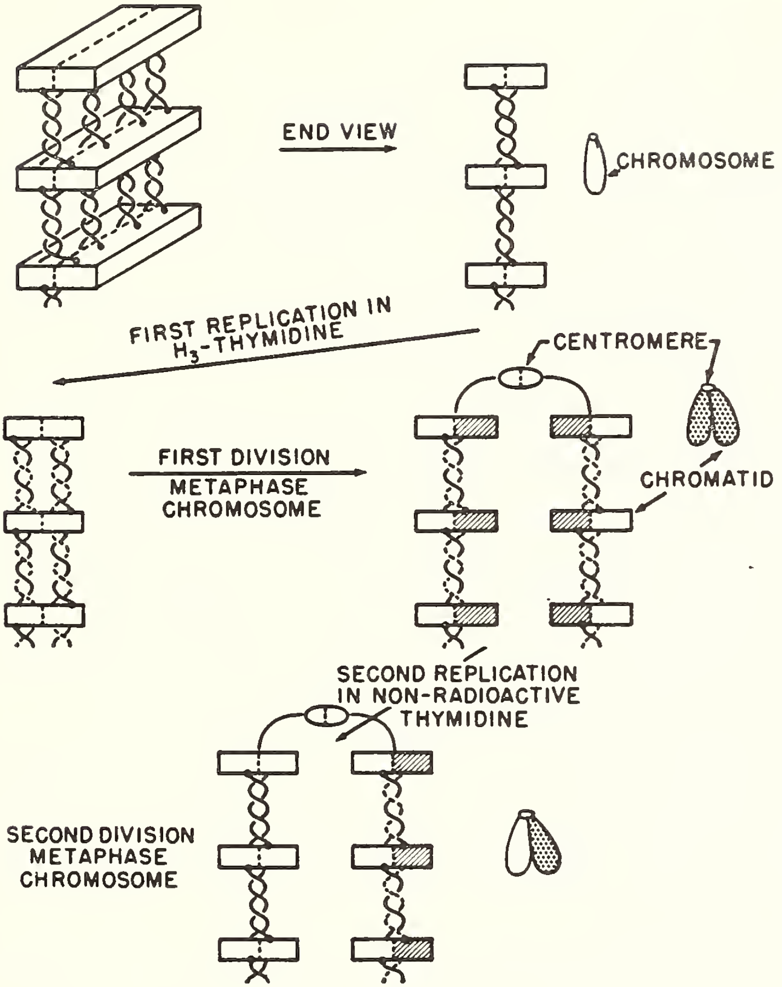

FIGURE 8.—A model illustrating that it is topologically possible for a multistranded chromosome to segregate labeled and unlabeled DNA at the second division following labeling. Four strands of DNA run from link to link along the length of the chromosome. *Heavy lines* indicate original DNA. *Dotted lines* indicate radioactive DNA.

DISCUSSION

Perry: The model you presented, or any model of this type requiring a small protein component which acts to keep the strands segregating together, might indicate that the protein component of the chromosome would segregate in the same way as the DNA component. The experiments of Prescott, showing that whereas DNA is semiconservatively replicated the protein is not, would not conform with this. Could you comment on why this might be fallacious.

Wolff: First, let me emphasize again that the model is not to be taken as an indication we think the chromosome is constructed that way. It is just to answer an argument I have heard many times that multistranded chromosomes cannot accommodate a semiconservative distribution of DNA. The model just shows, topologically speaking, we can think of schemes for this. Let me say one other thing. It is indeed true that Prescott's experiments show that the protein was dispersively distributed to the chromosomes. We know, however, from experiments in *Vicia* of Ellen Mattingly and those of others that although the protein part of the chromosome doubles the same time the DNA does, if we treat with radioactive amino acids we find incorporation not just in cells undergoing DNA synthesis, *i.e.*, are in the S portion cycle, but in 100 percent of the nuclei. This label is a labile one in that it leaches out. Later experiments by Prescott in *Amoeba* confirmed this. Therefore, we really do not have any idea as to what happens to that part of the protein that doubles when the protein is duplicated. In other words, there may be lots of proteinaceous material that may not be involved in the linear continuity of the chromosome. The part involved in the linear continuity might still be distributed semiconservatively or there might be turnover that would mask any basic semiconservative pattern.

Perry: Your technique of using trypsin combined with autoradiographic studies might be very useful to reduce this noise.

Wolff: Trosko has not done this yet for the protein part. He did try to do it for DNA because those strands (until you extract the DNA) are still Feulgen-positive and we thought we could see the distribution of label among quarter- or half-chromatids. It would be a good idea, I think, for him to try to check the incorporation with amino acids as you say.

Edgar: Would you care to comment on any evidence from a mutation argument.

Wolff: I could not do so with any great competence, but what we keep hearing at this meeting are references to cases of mosaicism. Peacock can tell you about the recent experiments of Novitski on mosaicism. Edington's experiments, too, can be easily accommodated with a multistranded chromosome. So there are genetic experiments that seem to fit the notion of a multistranded chromosome. We are, however, then left with the problem how mutations occur if there are extra strands of DNA (extra copies of genes). If there are many strands of DNA and one mutates, how come the others do not cover the mutation in terms of dominance? This is a great problem, but one that we may be forced to face. After all, as I said the other day, these chromosomes are not simply DNA molecules such as we see in *Escherichia coli* and in phage, and the problems may be much more complex in higher forms. It would be very nice if we could immediately make the jump from the phage and *coli* chromosomes to the great big clubs that I showed in the figure of metaphase, but we may not be able to do so.

Hoecker: I would like to have a clear idea about the matrix. It has been used so many times and in so many different ways.

Wolff: No, we have no real information on this, other than some treatments seem to cause it to disperse. Now, of course, cytologists for years have been talking about the presence of the matrix. If there is one and it is the dissolution of this matrix that causes the stretching and opening up, we would think it is nonhistone because pepsin, which removes histones, does not necessarily cause the dissolution of matrix. This is all we can say. We still are in the dark regarding the true nature of a chromosome, although you can see this gigantic structure with the light microscope, and in some cases, as with the lampbrush chromosome, for instance, it is almost visible with the naked eye. We still do not know how the molecules are put together and this is one of the great problems in modern biology.

Schultz: The problem that I cannot quite see through in these experiments is the following. What concerns us is whether there is a continuous DNA fiber which is really the basic element of the chromosome, the kind of thing we have as a model in the bacterial systems, but complicated by protein around it. Now, is it possible to imagine, in your experiments, for example, that the presence of the protein stabilizes the positioning of the DNA fiber in such a way that repair is facilitated and so the kinds of things that you have been observing would not be inconsistent with the continuous DNA structure in the chromosome. It seems to me that it is rather difficult to conclude from these rupture experiments the manner in which the structure itself has been put together. These templates that operate in a very delicately adjusted sequence of reactions give us a structure that we, of course, adopt as a stable thing, but of course the manner in which we can pull a thing apart does not necessarily tell us how it has been put together.

Wolff: Yes, but if the repair were in DNA alone, then we have to argue that it is only the disruption of the repair of the protein part of the molecule that causes the effect and not the disruption of the DNA part (because of the FUDR experiments). It is true that the formation of the links we make when we cause a rupture in DNA to come together may not necessitate DNA synthesis in the true sense but only a rejoining. We still do know, however, that DNA synthesis inhibitors do not affect repair, protein synthesis inhibitors do, and thus we have to argue in the face of this evidence. I admit that we cannot rule out that the repair is in DNA but does not require DNA synthesis. I think the formal possibility still exists.

Schultz: The reason I think this is important is a simple question of linkers versus nonlinkers. If you imagine that the repair of the linear continuity is a function of the protein, then you make unnecessary the picture of the chromosome in which you have a continuous code that is being read-off. It is from this point of view that I am concerned with your experiments, because if they can be made definitive they tell us a great deal but if they cannot then we have to continue floundering around.

Pavan: I think we are coming back to the work that was done 10 years ago when Darlington, Huskins, and others were discussing the number of threads in the chromosome. At that time, I remember I thought the misunderstanding or lack of agreement among these people could be accounted for on the basis that the work had been done with different materials. Now, you say that the chromosomes from germ line cells could have a different number of threads than those from somatic cells. Also, do you think it would be possible that different organisms would have a different number of threads in the chromosomes of corresponding somatic cells?

Wolff: Well, in view of what we know or what we have seen in the lampbrush chromosome, I would say perhaps anything is possible along these lines. In plants, however, we do not have a separate germ line. We have seeds that germinate and then vegetative cells eventually differentiate to form germ cells. As I understand it, most measurements have shown that the amount of DNA in a gamete is the haploid amount, and there is not the type of discrepancy you might expect if there were reductions in the numbers of DNA strands when gametes are formed.

Lara: I am very happy to see your model and Dr. Peacock's model. As far as I know, the best preparations of DNA obtained thus far do contain traces of amino acids and this might be part of the links that you have in your models. In this connection it will be very interesting to look for enzymes, which should hook up DNA to protein.

Streisinger: In connection with Dr. Pavan's question and your response to it, to what extent has the amount of DNA per nucleus in the cells that you have been cytologically examining been actually compared to the amount of DNA in a haploid component?

Wolff: In these particular cells I do not believe that any microspectrophotometric measurements have been made. I would refer to Dr. Moses here, to ask him if he knows whether any measurements have been made in *Vicia faba* gametes.

Moses: I cannot recall any such studies in *Vicia*. But in the root tips of *Vicia*, 2C, 4C, and 8C nuclei have been measured.

Streisinger: Then wouldn't one of the first orders of business be, in these cases that you are examining in great detail cytologically, to measure the amount of DNA in the very same samples?

Wolff: At one time we toyed with the idea of doing it and we were dissuaded by people who insisted that microspectrophotometry may not be accurate enough to detect such small differences. The errors in the method are too great. But I see Dr. Moses shaking his head "No" and perhaps we were dissuaded a little too easily.

Schultz: I should, in this context, mention some work that George Rudkin has been doing, that has been confirmed by Helen Gay. If one takes the metaphase chromosomes, many of us observed long ago that they have different sizes in different tissues and have wondered for a long time when we would get to measure them. Rudkin has been doing this and he finds that in the giant metaphases of the ganglia there is about twice the amount of DNA as there is in the embryonic nuclei of the salivary gland, which he has been investigating in detail. Gay found the same kind of thing, doing her work with measurements of Feulgen absorption. We have, I think, in *Drosophila*, quite good indications that the chromosomes may be polynemic. Of course this is an organism which is adjusted to polynemy. But, it does, it seems to me, open wide the possibility for support of the thesis of multistrandedness as a way of life, for mitotic organisms.

Reissig: If I may go back to the mutation evidence, I think it is not just that we find it more comfortable or more convenient to think of a single molecule, but it would be hard to interpret the data in terms of a multistranded structure. On the whole, the comparison of bacterial mutation data and Neurospora mutation data do not show any of the tremendous discrepancies we would expect if one structure is a single molecule and the other is a multiple molecule. I think the particular system I described in the first session could be used to exemplify the difficulties in thinking in terms of a multimolecular structure. In this system I was selecting mutants by virtue of the dominant property in a reduction of ATC on the suppression of argenine requirements. That is to say, it is a dominant selection mechanism and when you look at the mutants selected by this method a very appreciable proportion of these turn out to be complete deficiencies of ATC. On the other hand, there is no indication of mosaics if one takes care to avoid contamination by background conidia. This is a microconidial system. Therefore, it is very clear here that mutants obtained are not mostly partial but, on the contrary, are largely complete mutants, although the selective system is not biased in favor of them. From this I conclude that the DNA in Neurospora cannot be but one single molecule.

Wolff: In other organisms, of course, mosaicism has been found. I agree with you, indeed, that it would make life much easier for us if we did not have to contemplate multistranded chromosomes and that the gene did act as we know it does in systems where the gene is simply a DNA double-helix. But, you have presupposed here that when many genes are together they do not act as a unit but each acts individually. This might be true in microorganisms, but this may not be true in higher forms and, also, a bundle of genes may have only one *active* DNA molecule while the others are redundant or reserve. I know, it is very difficult for us, yet we have heard from other people here, *i.e.*, Dr. Schultz has mentioned Rudkin's work, of cases where multistrandedness seems to rear its head time and time again.

Hoffschneider: If I understood you, only protein synthesis is needed for the repair action, but do you really have proof that the morphological repair means also a functional repair of the chromosome.

Wolff: We do not know this because our studies have been strictly cytological and our one criterion has been whether we have a morphologically repaired chromosome. Whether there has been another step to make this functionally correct, I do not know because we have not made such experiments nor can I easily visualize this type of experiment. We do know that the chromosomes we get when we have translocations can be perfectly functional. Now whether there has been another chemical step to make them so has not been studied.

Miller: I am a nonbeliever. In the first place I do not think it fair, when you talk about multistrandedness in lampbrush chromosomes, to say that some believe in a single strand but others believe it may be multistranded. I think one should say that earlier observations suggested a multistranded structure in which the ribonucleoprotein matrix was considered as having continuity with the chromosome axis; however, recent experiments have conclusively shown that the earlier observations were erroneous in that the ribonucleoprotein matrix does not enter into the continuity of the chromosome axis. Second, the type of effect which you observe in *Vicia*, *i.e.*, that deoxyribonuclease will not break metaphase chromosomes, can be duplicated in the lampbrush system. That is, when we treat chromosomes in the lampbrush stage with deoxyribonuclease, they are readily broken. However, if we wait until these begin to approach diakinesis and then treat with deoxyribonuclease we cannot break this chromosome. I would interpret this as meaning that during coiling histone becomes cross-bonded so now the DNA can be taken out and leave a protein skeleton. Third, I belive that in the *Vicia* system you cannot demonstrate subchromatid division except by using surface film action, *i.e.*, these things are not visible until they are dried.

If I understand the technique correctly, Trosko was using trypsin digestion of *Vicia* chromosomes. If you observe these chromosomes with phase optics, you do not see any separation while they are in the liquid. You only see the separation after they are dried. I think this could as well be an artifact of the drying procedure as an indication of true subdivisions. May I make just one more point. As I asked Dr. Peacock yesterday, if you assume that the DNA is semiconservatively replicated and then you make the assumption of multistranded chromosomes, you are obligated, in addition to producing topological diagrams, to try to explain how segregation can occur on the molecular level.

Peacock: With respect to Dr. Miller's suggestion that the subchromatids visible in Trosko's preparations may be an artifact of the air-drying procedure, I think it should be explained that a very similar doubleness is seen in the *Vicia* chromatid after a variety of fixation procedures, without any air drying. More importantly, this same substructure has been found by Bajer in living cells of *Haemanthus*. Dr. Bajer has given me permission to mention this work. In his films of living mitotic cells of *Haemanthus*, Bajer demonstrated clearly visible half units in the anaphase chromatid: These half-chromatids resemble those of the fixed and air-dried preparations mentioned above. Bajer also found that ultraviolet microbeam irradiation of the centromere region often produces two well-separated strands in the centromere region. He also noted that subchromatids are often spatially separated in regions of the chromatid which are demonstrating neocentric activity. I think we must conclude that multiple strandedness has been demonstrated both *in vivo* and in various fixation procedures. I hasten to add that this does not necessarily demand that genetically identical molecules are repeated in each of the strands.

Streisinger: Some of the results presented by Dr. Peacock and by Dr. Miller yesterday seemed to me at first to disagree with some of the results presented by Dr. Wolff.

Dr. Wolff suggested that the chromosome structure was *not* destroyed by deoxyribonuclease treatment but was "demolished" by long incubation with trypsin; Dr. Miller, on the other hand, presented results suggesting that deoxyribonuclease treatment cleaved chromosome strands, whereas trypsin treatment did not destroy them.

The contradiction, I believe, is not real but only apparent. I wonder whether others, who may be as ignorant and naive with respect to this field as I am, would be helped to a clearer understanding if those working in this field would try to differentiate between various levels of organization of the material that they may be studying.

Instead of speaking of *the* "structure of the chromosome" a *particular* level of organization or structure ought therefore to be referred to.

I would like to suggest that the most extended form of the chromosome be referred to as the *primary structure*. Operationally, one would eventually like to refer to the primary structure as the one held together exclusively by covalent bonds.

The chromosomes present in interphase nuclei may perhaps possess relatively little tertiary structure and, thus, may most clearly show primary structure.

Various more highly organized regions of the "fully extended" chromosome that contain loops or rings at defined sites could be referred to as exhibiting *secondary structure*.

The highly coiled form of the chromosome, consisting of an association of DNA, RNA, and protein, could be usefully referred to as the *tertiary structure*.

With these considerations the apparent contradictions that I have mentioned disappear. Dr. Wolff's results suggest that the tertiary structure of the chromosomes that he is studying is destroyed by trypsin treatment but not by deoxyribonuclease. This observation is important with respect to understanding the tertiary structure of the chromosome but is not relevant to understanding the primary structure. Dr. Miller and Dr. Peacock, on the other hand, seem to be examining chromosomes that may possess relatively little tertiary structure and thus give information that is relevant to an understanding of their primary structure.

Dr. Peacock (and others) suggested that chromatids may be composed of several strands. It may be useful, in these cases, to refer to a *quarternary structure*.

Cooper: May I ask Dr. Streisinger how he recognizes any of these different levels operationally? It is all well and good to say that the chromosome ought to have these four levels, and perhaps more. The primary structure in itself is an hypothesis. One way of defining the nucleus, for example, is that it is the sum of the chromosomes in their interphase stage. We do not know, for instance, that we speak with acuity when we talk about a nucleus and ask where the chromosomes are in it. The nucleus is the chromosomes and their products at interphase. How, operationally, should we go about using your recommendations?

Streisinger: I would like to emphasize that I am utterly ignorant with respect to the things that I am talking about.

One way of defining the primary structure would be to consider it as the axis that is covalently bonded throughout. This may be experimentally difficult to distinguish; nevertheless, I think it may be useful to try to think in terms of identifying this structure and determining the properties of it. The interphase chromosome certainly looks very different from the highly coiled metaphase chromosome and it would be useful to try to determine whether its properties would be such as to suggest that it contains only primary structure.

Cooper: They look different, but we do not know that they have lost anything or gained anything. In other words, you have stated a certain hypothesis when you say they are very different. There are organisms, for example, which have magnificent chromosomes for which there seems never to be an interphase structure, for the chromosomes are condensed at all times, even when, presumably, they are doing whatever work they do metabolically. I am thinking now of the Hypermastigine protozoans that Professor Cleveland has worked on. If these represent the chromosomes in all their stages, without great force of logic, one might conclude everything that makes these chromosomes comprises a very complex primary structure. I would not know operationally, as a cytologist, how to talk about "primary structure" except in the most hypothetical terms at this stage of the game.

Streisinger: I do not want to defend these proposals too much and they should certainly not be taken too seriously. I merely wanted to suggest that it may be useful to indicate the level of organization of the structures being considered and that a nomenclature such as the one I suggested might help us to do that.

As I had said earlier, the destruction of the structure of the chromosome by trypsin may imply, in a particular case, the destruction of tertiary structure and this is very different from the destruction of primary structure. To illustrate this a bit further: It is very useful to inquire into the role of protein molecules in maintaining tertiary structure and also to ask whether DNA is covalently bonded to protein molecules in a continuous axis (in a primary structure) but these two questions are very different. An ignorant outsider like myself would be helped in the understanding of which of these problems is being discussed by the adoption (if and where possible) of the kind of terminology that I suggest.

Moses: I think Dr. Streisinger's suggestion might be helpful to some of us. One thing that has concerned me, and I have said this on other occasions, is the confusion arising in the use of the term "strand." I probably have been guilty of misusing the word strand when talking about chromosome organization. A strand may mean anything from the primary level of structure to the quaternary level when we are discussing chromosomes. We may speak of a strand as a polynucleotide chain, a single strand of DNA. We may say "DNA strand" and think of a chain of DNA (double helix) molecules; we may think of a strand as being the axis of the lampbrush chromosome. We may think of strands as being the protein structure that Dr. Wolff talked about. Strands may also refer to something even more coarse than this. I think it is first necessary to recognize and define the level of chromosome organization we are talking about. We must keep in mind that "the chromosome" is not a metaphase, meiotic, or lampbrush chromosome. It is a structure which is able to transform from one to the other of these various states of organization. It can pass from interphase through division and even reduce itself to a minimal amount of material in the sperm. But there must be some primary structure that is a common denominator. From these transformations we can perhaps set up an hypothesis of what it is, and possibly what a common secondary structure may be. As a way of sorting out our thoughts, thinking in terms of a systematic hierarchy of organization is perhaps useful.

Wolff: This is what we are striving to find. We want to know what the chromosome's structure is. That is the purpose of all these experiments and, as yet (as the comments made during the last few days show), I do not think we have the answer.

Modification of Breakage and Reunion in Relation to Lethal Damage [1, 2]

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SUMMARY

The modifications induced by metabolic inhibitors, given before or after X irradiation on mitotic delay, chromosome breakage, and cell lethality, were studied in a cell population of ELD Ehrlich ascites tumor. The generation time of this tumor is 28 hours. G_2 lasts 6 hours, S is about 13 to 15 hours, G_1 is about 6 to 8 hours, and the mitotic time is 0.75 hours. After irradiation with 500 r, a mitotic delay of 6 hours, mainly due to a block in G_2, was found. FUDR alone prolonged the time of S, did not delay G_2, and the combined treatment—FUDR plus 500 r—induced both a block in G_2 and prolongation of S. Chloramphenicol or sodium azide alone delayed the cells in G_2, but did not increase significantly the mitotic delay over that induced by X rays alone. Chromosome breakage induced by 500 r was increased by pretreatments or posttreatments with inhibitors, depending on the stage of the cell cycle. For instance, pretreatment with sodium azide increased the frequency of breaks all over the mitotic cycle, but the maximum effects were seen in middle G_2 and early S. Pretreatment with chloramphenicol increased breakage, mainly in G_2 and late S, which indicated more protein synthesis would be necessary for rejoining during those periods. Pretreatments or posttreatments with FUDR increased breakage in cells from G_2, S, and G_1, which is considered an indication that this effect is related to processes other than deoxyribonucleic acid (DNA) synthesis. Lethal damage was also increased by some inhibitors, but this effect was not always seen in the same stage of the cell cycle at which increased breakage was observed. When the dose of X rays was 500 r, none of the inhibitors modified the percentage of tumor takes in the animals, but the cell population decreased in the first hours after irradiation. With this dose of X rays, pretreatment with sodium azide killed cells mainly from S, chloramphenicol damaged cells from G_2 and perhaps from G_1, and posttreatment with FUDR was lethal for many cells from G_2 and S. When the inhibitors were used with 2000 r, pretreatment with sodium azide and the combined pretreatment and posttreatment with chloramphenicol killed the entire cell population. FUDR given at high concentrations after irradiation damaged a high proportion of the cell population and tumor growth was inhibited in 80 percent of the animals. This last effect was not reversed by thymidine or uridine and therefore does not seem to be due to inhibition of DNA or ribonucleic acid synthesis. These findings lead us to postulate that

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] This investigation was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

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mitotic delay, chromosome breakage, and cell lethality are three independent processes not produced by the same mechanism. We also conclude that the lethal damage induced in a cell population is due only in part to chromosome damage, and that another part is caused by the inactivation of other target molecules, related or not to DNA synthesis.—Nat Cancer Inst Monogr 18: 181–212, 1965.

IT IS COMMONLY accepted that cell death induced by ionizing radiation is mainly due to nuclear damage, but no simple correlation has been established between the amount of lethal damage and the frequency of chromosomal aberrations. In early works Puck *et al.* (*1*, *2*) postulated a two-hit aberration survival curve for mammalian cells in culture, but later on (*3*) he included other types of chromosomal damage as possible causes for cell death after irradiation. More recently it was proposed [Puck and Yamada (*4*)] that visible chromosomal aberrations, cell death, and mitotic delay are different expressions of the same principal effect, namely, the breakage of the deoxyribonucleic acid (DNA) molecule. This correlation was not found by Elkind (*5*) who showed that mitotic delay is independent of cell death and proposed that there is lethal damage only when some essential substances are no longer synthesized or are present in an amount insufficient for growth. Stapleton (*6*), Alper (*7*), and Kimball (*8*) showed that nutritional and physiological factors modify the lethal effect induced by ionizing radiation. Their action would be related to the expression of the initial damage and to repair mechanisms [Thornley (*9*)]. Since Wolff and Luippold (*10*) demonstrated that metabolic conditions are involved in the process of rejoining of chromosome breaks, then cell death could also be the result of increased frequency of chromosomal aberrations, produced by inhibition of restitution.

The interrelationships between primary chromosomal damage, metabolic effects, mitotic delay, and cell death are complex, and the mechanisms involved remain obscure. In an attempt to explore these interrelationships, we have performed a series of experiments using different temperatures and metabolic inhibitors as pretreatments or posttreatments with a single dose of X rays.

The effects of these inhibitors on chromosome breakage, mitotic delay, and cell death were analyzed by a procedure enabling us to study all effects in the same cell.

PROCEDURE DEVELOPED FOR THE STUDY OF CHROMOSOME ABERRATIONS, MITOTIC DELAY, AND CELL LETHALITY IN THE SAME CELLS

Many investigations on chromosome breakage, mitotic delay, and cell death induced by X irradiation have been published in recent years. Most of them, however, deal with only one of these effects, though some relate chromosome breakage and mitotic delay. Only very few *(11)* attempted to correlate the three effects in the same cell, because the material and methods used did not permit the study of chromosomal damage when cell survival was determined. To avoid this problem, we developed a new procedure, which combines counting the number of cells present at different times after irradiation, with the cytological techniques for mammalian chromosomes. The tumor is treated *in vitro,* and a known number of cells are injected into each animal which is killed at different times after irradiation, and the total number of cells per animal determined. These values are taken as a measure of the number of surviving cells. Some dying cells, of course, can be counted with nondamaged cells and one cell count does not show exactly the fraction of surviving cells, but if we make several counts, dead cells soon disappear from the cell population. Moreover, the division of undamaged cells could increase the total number of cells despite the death of a fraction of the cell population. What we are measuring, therefore, is the dividing ability of the surviving fraction of a cell population. This measure gives a good indication of the proportion of lethal damage induced by the treatment.

While studying lethal damage by this technique, we can use the same cells for cytological studies. The type and frequency of chromosome aberrations can be scored with great accuracy, comparable to that described for *Vicia faba* or hamster cells in culture.

Not using colchicine to accumulate mitoses, we can study the mitotic index in the same slide. Autoradiographs can also be made with the same material when a pulse of tritiated thymidine is given before or during the treatment.

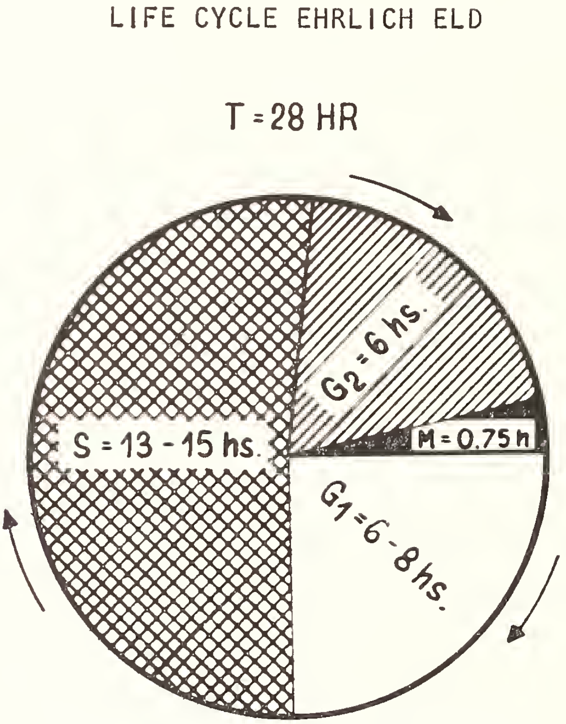
The procedure has been described in detail previously *(12, 13)*. Briefly, it consisted in the irradiation *in vitro* of 5 ml of ELD Ehrlich ascites tumor, harvested from animals inoculated 8 to 10 days before. After irradiation 20×10^6 cells were inoculated intraperitoneally into each of 20 to 30 C3H/Ep mice, 2 to 3 months of age. Thereafter, 1 to 2 mice were killed at each chosen interval after irradiation and the total number of cells per animal determined by the method of Klein and Révész *(14)*. For each treatment, 10 to 15 animals were used to study the percentage surviving without tumor, and each experiment was repeated 6 to 8 times. The results presented are the mean of the values obtained for each experiment. With this data, growth curves were obtained and used as a measure of the proliferation of the irradiated cells. For cytogenetical studies, the cells were treated with hypotonic solution (Ringer-water 1:5) and fixed with Carnoy's or alcohol-acetic acid 3:1. Smears were air-dried and stained with Unna's alkaline methylene blue, after hydrolysis with normal hydrochloric acid.

For the mitotic delay, the mitotic index based on 1,000 cells per smear and the percent of labeled mitoses were determined in autoradiographs after pulse label of 200×10^6 cells during 20 minutes with 50 μc of tritiated thymidine (fig. 1). Eastman Kodak NTB emulsion *(15)* was used, with an exposure time of 10 days, and the autoradiographs were stained with

Unna's alkaline methylene blue, without hydrolysis. We determined the percentage of labeled metaphases on 100 cells, observing if all the chromosomes, only some of them, or regions of the chromosomes were labeled.

TIMING OF MITOTIC CYCLE (UNIRRADIATED)

In pulse-labeling experiments carried out in collaboration with Dr. T. C. Negrotti (*16*), a generation time (T) of 28 hours was found for the ELD ascites tumor cells. The doubling time measured from growth curves also appeared to be the same (text-fig. 1).



TEXT-FIGURE 1.—Life cycle of the ELD Ehrlich ascites cells.

The time for reaching 50 percent labeled metaphase was 6 hours and 45 minutes, and the period of mitosis (M), as measured by the method of Stanners and Till (*17*), was 45 minutes. Therefore, the G_2 period was 6 hours (6 hr. 45 min–45 min).

The period of DNA synthesis (S) appeared to be 15 hours when measured as the time during which more than 50 percent labeled metaphases were seen, and was calculated to be 13 hours, by the method of Stanners and Till (*17*). Finally, a G_1 period of 6.3 to 8.3 hours was obtained by subtracting from T (28 hours) the periods $M + G_2 + S$.

EFFECT OF X IRRADIATION

Mitotic Index

After irradiation with 500 r at 20°, the mitotic inhibition lasted for 6 hours and the mitotic index returned to the control values at 12 hours. A first peak of mitotic activity (130% of the control) was observed at 16 hours and a second peak at 40 hours.

Percentage of Mitoses With Labeled DNA (*In Vitro* Labeling)

After irradiation with 500 r at 20°, the time for appearance of 50 percent labeled mitoses was 12 hours, with a prolongation of G_2 of about 6 hours. The S period was not prolonged, and G_1 cells were not delayed. The highest percentage of labeled mitoses found was 80 instead of 100 percent, probably because the other 20 percent were delayed G_2 cells which reached metaphase together with the cells in S. It is also possible that X irradiation inhibits DNA synthesis in some of the cells in S during the irradiation.

Chromosome Breakage

X irradiation with 500 r induced 1.3 mean breaks per cell in the unlabeled cells fixed 10 hours after irradiation. At 12 hours, the mean number of breaks was 2.6 in unlabeled metaphases from G_2 and 1.5 in the labeled cells from S. At 16 hours it was 2 in unlabeled cells and 1.1 in labeled cells, at 24 hours it decreased to 1.3 and 1, respectively, and at 26 hours it was 0.87 in unlabeled cells from G_1. In the samples fixed at 30 hours, the metaphase represented the second division of cells treated during mitosis and showed stickiness instead of chromosome breaks. The anaphase presented multiple bridges and fragments.

Cell Lethality

After 500 r at 20° no significant decrease in the number of cells was found. The cell population increased slowly during the first 12 hours, in which an inhibition of the mitotic activity was observed. After that time, a logarithmic growth similar to the one described for the untreated control was observed. Lethal damage to the cell population was not detected, therefore, by the study of the growth curves. Nevertheless, the cells with chromosome aberrations, observed during the first 48 hours, disappeared from the cell population afterwards. They are lethally damaged cells that can undergo only one or two divisions, but they represent a small proportion of the cell population whose elimination does not modify the growth curve of the tumor.

Tumor growth was inhibited by 2000 r during the first 6 to 8 days, after which growth was logarithmic. All the animals died with tumor 15 to 20 days after inoculation of 20×10^6 treated cells. The period of inhibition of the tumor was due to the death of many cells, some of them after one or several divisions and the others in the first division after a long period of mitotic delay. During this period the number of undamaged cells increased and restored the logarithmic growth, while all the lethally damaged cells disappeared from the population.

EFFECT OF TEMPERATURE *IN VITRO*

Since irradiation and treatment with metabolic inhibitors were carried out *in vitro*, it was important to know if the temperature itself could modify the results. To test this, the tumor was kept *in vitro* at 37° and the results compared with those observed at room temperature (20°C). The following modifications were observed.

Mitotic Index

When X irradiation with 500 r was given after the tumor was kept *in vitro* for 1 hour at 37°, mitotic inhibition was increased up to 12 hours. The mitotic index reached the control value at 18 hours, and a very high accumulation of mitoses (230% of the control) was observed 24 hours after irradiation.

Percentage of Mitoses With Labeled DNA (*In Vitro* Labeling)

After pretreatment for 1 hour at 37° and X irradiation with 500 r, the time for reaching 50 percent labeled metaphase and the S period were not significantly prolonged, but the highest percentage of labeled metaphases was 60 percent at 16 hours. This low percent of labeled mitoses might be due to a reduction in the synthesis of cells in S during the treatment at 37°, or to accumulation of G_2 cells with a long mitotic delay, which reached metaphase with the nondelayed or slightly delayed cells in S during irradiation.

Chromosome Breakage

Pretreatment for 1 hour at 37° not only increased the mitotic delay in G_2 and the subsequent increase in mitotic activity induced by 500 r, but it also increased the frequency of breaks in those cells accumulating 24 hours after irradiation. At that time, the mean number of breaks per cell was 2.7, while with 500 r at 20° it was only 1.3.

When the pretreatment at 37° lasted 3 hours, the frequency of breaks was even higher, and the mean number of breaks per cell at 24 hours was 4.7.

Cell Lethality

When the ascites tumor was maintained *in vitro* for 1 hour at 37° before X irradiation with 500 r, the number of cells decreased slightly up to 16 hours but then resumed logarithmic growth. As mitotic inhibition occurred during the first 12 hours, and only a small proportion of cells were lethally damaged, the type of growth of the entire cell population was not modified.

Four hours at 37° before 500 r decreased the number of cells during the first 24 hours, with an increase in the proportion of damaged cells, but the effect did not modify the growth of the entire cell population.

Pretreatment at 37° for 1 hour increased the period of tumor inhibition when the X-ray dose was 2000 r. The number of cells remained constant until the 15th day, the logarithmic growth was seen from the 15th to the 20th day, and all the animals died with tumor during the 20th to 25th day after inoculation.

Tumor kept at 20° before irradiation with 2000 r was inhibited for only 8 days, which indicated that the percentage of damaged cells was increased when the tumor was stored at 37° before X irradiation. This increase of lethal damage was highest when the cells were maintained for 4 hours at 37° before irradiation with 2000 r. Under these conditions, the whole cell population was killed and there was permanent inhibition of tumor growth.

In view of these results, a constant temperature of 20° was employed, except when testing whether the effect of the inhibitor was modified by a different temperature.

EFFECT OF METABOLIC INHIBITORS

The metabolic inhibitors used in the following experiments were selected because their main action interferes with some important synthetic processes of cell metabolism.

5-Fluorodeoxyuridine is an inhibitor of DNA synthesis, and generally does not modify the synthesis of ribonucleic acid (RNA) and proteins, though there is some evidence [Volkin (*18*) and Lindner (*19*)] that other effects are induced. Chloramphenicol and puromycin are well known as inhibitors of protein synthesis, and sodium azide is an inhibitor of oxidative phosphorylation, which is an important step in the production of the energy necessary for cell respiration and other synthetic processes.

The object of these studies was to obtain some data on the metabolic processes involved in the expression of radiation damage and to determine whether the modification in cell metabolism changes the sensitivity of the cells to the primary action of X rays or affects the mechanisms of repair.

For each inhibitor, experiments comparing the effect of pretreatment, posttreatment, or both were made. An increased effect only after posttreatment would mean the synthetic process inhibited is necessary for repair. But if pretreatment only produces an effect, it would be because the action of the inhibitor leads to some metabolic deficiency that increases the sensitivity of the cells to the action of X rays.

Pretreatment or posttreatment that similarly increases the effect of radiation might be due to an inhibition of metabolic processes necessary for repair. Posttreatment increasing the effect of pretreatment might be an indication that both increased sensitivity and interference with repair are involved.

Some data on the effect of the inhibitor without irradiation are also described, to establish if the damage observed after the combined treatment could be explained by the addition of two independent effects.

EFFECT OF FUDR ALONE

A treatment during 1 hour with FUDR (4×10^3 M) at 20° induced the following modifications.

Mitotic Index

The mitotic index was low during the first hours after treatment, but a complete inhibition of mitosis was never found. The values decreased during the first 4 hours, reaching 60 percent of the control, and remained the same from 4 to 24 hours. They were similar to the untreated control at 40 hours.

Percentage of Mitoses With Labeled DNA (*In Vitro* Labeling)

The time of appearance of labeled metaphases was similar to that observed in the untreated control, and 100 percent labeled metaphases were seen at 14 and 16 hours. This is considered as an indication that G_2 and late S cells were not delayed when treated for 1 hour with FUDR.

There was, however, a prolongation of the S period, indicative of a slow rate of synthesis, in those cells in early or middle S when the FUDR was added.

The absence of inhibition of DNA synthesis in cells at the end of the S period was probably because the thymidine already synthesized was enough for completing their synthesis, while the lack of thymidine induced by FUDR stopped DNA synthesis in the cells of middle or early S. Since FUDR was removed after 1 hour, the inhibition of DNA synthesis was only temporary and the cells began again to synthesize DNA, but the synthesis proceeded slowly until enough thymidine was available.

Chromosome Breakage

5-Fluorodeoxyuridine induced chromosome breakage in unlabeled cells from G_2, fixed 4 hours after treatment. At that time the frequency of breaks was low (0.34), but increased up to 0.94 at 24 hours and 1.4 at 30 hours. At 24 and 30 hours they were observed in labeled cells from middle or early S, delayed by FUDR. At 40 hours the frequency of breaks was 0.94 and most were in unlabeled G_1 cells.

The aberrations were predominantly of chromatid type, especially isochromatid breaks and chromatid exchanges, in all the samples studied, including the last one in which a high proportion of the cells were in G_1.

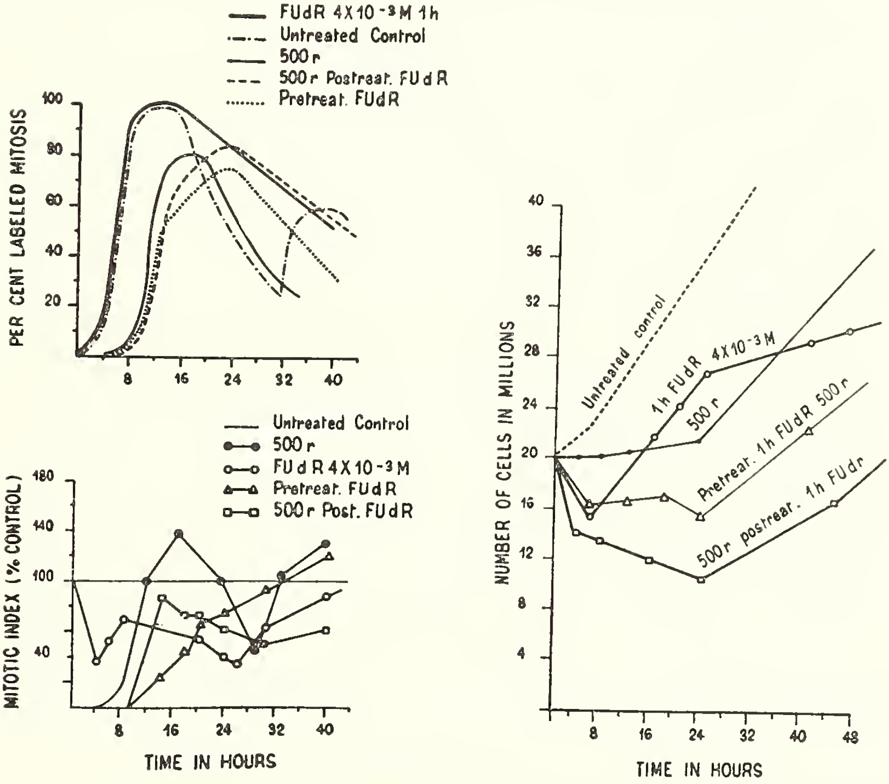
Interestingly, FUDR seems to induce breakage in cells in G_2 or G_1, as well as in S, which indicates that it can break the chromosome in the absence of DNA synthesis.

Cell Lethality

The number of cells decreased slightly during the first 8 hours. After that time, the cell population increased at a rate slower than the control, probably due to the low mitotic index described above.

EFFECT OF FUDR AND X RAYS

When the treatment with FUDR was administered before and/or after irradiation the following alterations were observed (text-fig. 2):



TEXT-FIGURE 2.—Effects of FUDR 4 × 10^{-3} M given alone during 1 hour (○–○), as a pretreatment of 500 r (△–△), or as a posttreatment after 500 r (□–□) on: 1) the percent labeled mitoses (*upper left*), 2) the mitotic index (*lower left*), and 3) the number of cells per animal, measured as a function of time after X irradiation. Cells were labeled *in vitro* for 20 minutes before X irradiation and followed *in vivo* up to fixation.

Mitotic Index

For pretreatment, or posttreatment, or both, with FUDR and 500 r, a mitotic inhibition of 8 to 10 hours was found. After the posttreatment the mitotic index reached a value of 80 percent of the control 16 hours after irradiation, and remained as 60 to 80 percent of the control from 20 to 40 hours. After pretreatment, there was an increase up to 80 percent at 20 hours, which reached the control values at 32 hours.

These results indicate that after a combined treatment of FUDR and X rays the mitotic inhibition is 2 to 4 hours longer than the one induced by 500 r alone; the percentage of cells reaching metaphase in 16 to 40 hours is lower than the control and there is no peak of mitotic activity similar to the one observed after 500 r alone.

Percentage of Mitoses With Labeled DNA

After combined treatment very few unlabeled G_2 cells reached metaphase, while labeled cells from late S appeared as soon as the mitotic index began to increase. Labeled cells reached a value of 80 percent of the control at 24 hours, which is a percentage similar to that found after 500 r alone. This indicates that in late S FUDR did not increase the inhibition produced on DNA synthesis by X rays alone.

From 24 up to 40 hours the proportion of labeled metaphases decreased slowly and reached approximately 50 percent at 40 hours. This prolongation of the S period induced in the cells in middle or early S was similar to the one described after treatment with FUDR alone and was not observed after 500 r alone.

Chromosome Breakage

Pretreatment for 1 hour with FUDR at a concentration of 4×10^{-5} M or 4×10^{-3} M, carried out at 20° or 37° (*20*), tripled the mean number of breaks induced by 500 r in the cells fixed at 12 and 16 hours (cells of G_2 and late S). The frequency of breaks increased up to 4.3, which is 4 times higher than the value with 500 r at 24 hours.

Most of the cells fixed at 24 hours were from middle S. At 40 hours 55 percent were labeled cells from early S and 45 percent unlabeled cells from G_1.

A high frequency of chromatid-type aberrations was observed in all the samples, including those containing cells from late G_1. For example, many chromatid exchanges (113.3/100 cells), isochromatid breaks (486.6/100 cells), and chromatid breaks (33.3/100 cells) were found either in labeled or in unlabeled cells fixed at 40 hours.

Posttreatment for 1 hour with the same concentrations of FUDR did not increase the frequency of breaks in the cells fixed at 16 hours (G_2 and late S), with a mean number of breaks of 1.7, but produced a number of breaks as high as those described for pretreatment at 24 hours (4.5 in cells of middle S) and a frequency of 5.1 at 40 hours (cells from early S and G_1). The predominant aberrations were again of chromatid type and were either in labeled or unlabeled cells.

Pretreatment with FUDR for 1 hour, 500 r, then posttreatment with FUDR for 1 hour gave results similar to those described for pretreatment alone.

In summary, *pretreatments with FUDR increased the sensitivity* of the cells in G_2 and late S to breakage induced by 500 r, while no effect was seen with posttreatment. For cells in S or late G_1, either the pretreatment or posttreatment increased 4 times the frequency of breaks as after 500 r alone.

When thymidine and uridine, at a concentration of 4×10^{-3} M, were added with FUDR, a decrease in the frequency of breaks was observed at all the times of fixation studied. This concentration of thymidine and uridine reversed in the same form the chromosome-breaking effect of either 4×10^{-5} M or 4×10^{-3} M concentrations of FUDR. The mean number of breaks decrease, being similar to the number induced by X rays alone at 16 hours, and was considerably reduced at 24 and 40 hours, though at these times it was still higher than the irradiated controls.

In other experiments, thymidine and uridine were added after 500 r, preceded by a pretreatment with FUDR for 1 hour. In this instance, the amount of breakage was reduced to values similar to those seen at 16 hours in the irradiated control. The frequency of breaks was not modified at 24 hours and was reduced to only half at 40 hours.

These results indicate that a reversion was induced for cells in G_2 and late S, even when the thymidine and uridine were given after X irradiation. A lower but similar effect was observed with cells in G_1 and early S, but for cells in middle S, a reduction in the frequency of breaks was seen only when thymidine and uridine were added with FUDR. This might be explained if more thymidine is necessary for repair of the breaks induced during middle S, or if thymidine competes with FUDR, preventing its sensitizing effect, when given at the same time, but not when added after X irradiation.

Apparently, FUDR increases the frequency of breakage induced by X rays through two different mechanisms, one related to DNA synthesis and the other to some unknown process which takes place *also during G_2 and G_1*. Since thymidine and uridine revert FUDR effect in different moments of the mitotic cycle, acting on one or the other mechanisms of breakage, new experiments are now in progress to determine if the reversion induced in G_2 and G_1 depends on the presence of uridine and therefore could be related to RNA synthesis.

Cell Lethality

After pretreatment for 1 hour with FUDR (4×10^{-3} M) at 20°, followed by 500 r, the number of cells slightly decreased up to 16 hours, remained the same for 16 to 24 hours, and began to increase after 24 hours, reaching at 32 hours the same number inoculated at time 0.

Posttreatment with FUDR (4×10^{-3} M) at 20°, given after 500 r, reduced the cell population to nearly half during the first 20 hours. After 24 hours, the number of cells began to increase slowly, reaching at 50 hours the same number inoculated immediately after irradiation.

This reduction in the number of cells seen during the first 16 to 20 hours, which is the time of mitotic inhibition, and the appearance of labeled metaphases as soon as mitoses began to appear, are considered as indications that many G_2 cells died without reaching metaphase. However, most of the cells in S or G_1 during treatment were able to reach division after mitotic delay, and though some of them died afterward, due to chromosome aberrations, others restored the growth of the cell population.

The experiments with 500 r indicated death of G_2 cells, due to inhibition of cell division, was more pronounced when FUDR was given as posttreatment than as pretreatment. However, cell death affected only a small fraction of the cell population and did not modify the development of the tumor. For that reason, in other experiments a higher dose was used (200 r). This does not itself reduce the percentage of tumor takes but the increased lethality induced by FUDR could be measured by the inhibition of tumor growth (text-fig. 3).

The results showed that pretreatment for 1 hour with FUDR (4×10^{-5} M or $4 \times {}^{-3}$ M), at 5°, 20°, or 37°, did not increase significantly the lethal damage induced by 2000 r. All the animals inoculated with these pretreated cells died with tumor within 25 to 30 days of inoculation (74% with ascites tumor and 26% with solid tumor developed at the site of inoculation). On the other hand, posttreatment for 1 to 3 hours with FUDR (4×10^{-5} M at 37° or 4×10^{-3} M at 5°) increased the lethal damage induced by 2000 r. Thirty percent of the animals inoculated with the posttreated cells survived without tumor for more than 6 months. Posttreatments with FUDR (4×10^{-3} M) for 1 to 3 hours at 20° or 37° induced an even higher lethal effect, 80 percent of the animals surviving without tumor more than 6 months. The remaining 20 percent developed a solid tumor and died 2 to 5 months after the inoculation.

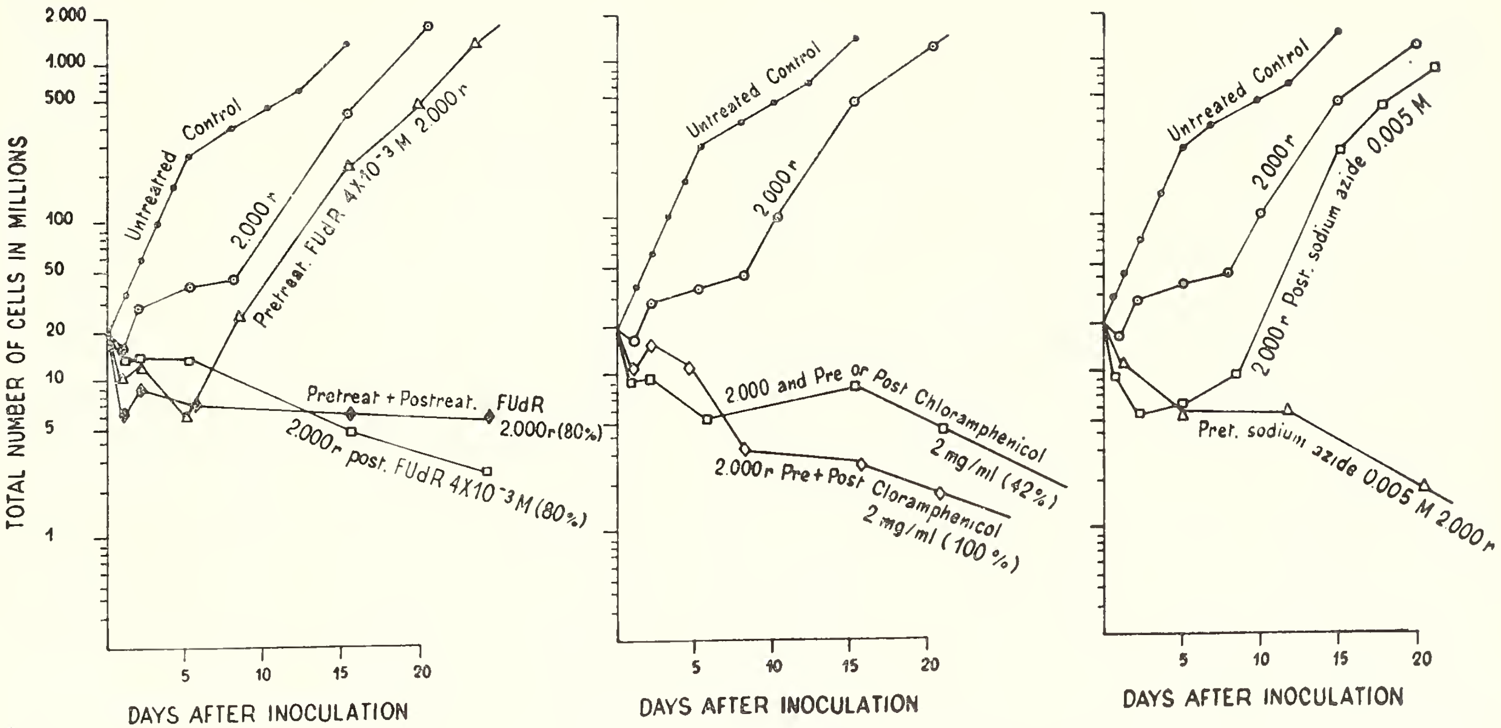
Combined pretreatment and posttreatment produced an inhibition of tumor growth similar to that induced by the posttreatment alone.

The addition of thymidine (4×10^{-3} M or 4×10^{-2} M) alone or together with uridine (4×10^{-3} M) did not reverse this lethal damage.

These results indicate that the lethal damage observed after 2000 r and posttreatment with FUDR became greater with the increase in the concentration of FUDR used, was higher at 37° than at 20° or 5°, and was not reverted by the addition of thymidine alone or together with uridine. Thus, although a great proportion of the cell population died when FUDR was present after irradiation, this lethal damage does not seem related to inhibition of DNA synthesis. It could be due perhaps to interference with another metabolic process necessary for cell survival.

EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS

Although low concentrations of chloramphenicol inhibit protein synthesis in bacteria, high concentrations must be used to obtain inhibition of amino acid incorporation in intact mammalian cells. According to Weisberger and Wolfe (*21*), the mechanism of inhibition is the same in



TEXT-FIGURE 3.—Growth curves after pretreatment, or posttreatment, or both, with FUDR 4 × 10^{-3} M, chloramphenicol 2 mg per ml, or sodium azide 0.005 M and 2000 r. Treatment done *in vitro* at 20° and cells grown *in vivo* in C3H mice.

both systems and the difference in sensitivity may be related to differences in template RNA turnover and stability. Since we have used a high concentration of chloramphenicol (2 mg/ml), the effects we describe here are considered to be related to the inhibition of protein synthesis.

To confirm these results we have repeated the same experiments with puromycin (1 mM), an inhibitor of protein synthesis (*22*), acting, both in animal (*23*) and microbial preparations (*24*), on the transfer of amino acyl residues from sRNA into long-chain polypeptides. Preliminary results show that both chloramphenicol and puromycin similarly modify the effect of radiation. But we will describe in detail only the effect of chloramphenicol.

EFFECT OF CHLORAMPHENICOL ALONE

After treatment of 200×10^6 cells for 1 hour with 2 mg per ml of chloramphenicol, we observed the following results.

Mitotic Index

During the first 8 hours the mitotic index decreased to 30 percent of the control and then increased to control values at 16 hours. A peak of accumulation of mitoses, with values of 140 to 150 percent of the control, was found from 16 up to 28 hours, which indicates that delayed cells from G_2 reached metaphase together with nondelayed cells from S and G_1.

Percentage of Mitoses With Labeled DNA (*In Vitro* Labeling)

The presence of nearly 20 percent labeled metaphases from 8 to 10 hours after treatment, when the mitotic index reached its lowest value, indicates that mitotic delay was mainly induced in G_2 cells. The length of the S period was not prolonged and the highest percentage of labeled metaphases was 60 percent. Since the percentage of labeled interphases was increased instead of decreased, the low percentage of labeled metaphases seems due to the accumulation of delayed unlabeled cells from G_2, delayed and nondelayed labeled cells from S, and nondelayed unlabeled G_1 cells, reaching metaphase together in the samples fixed at 16 up to 24 hours.

Chromosome Breakage

The treatment with chloramphenicol alone did not induce chromosome breakage.

Cell Lethality

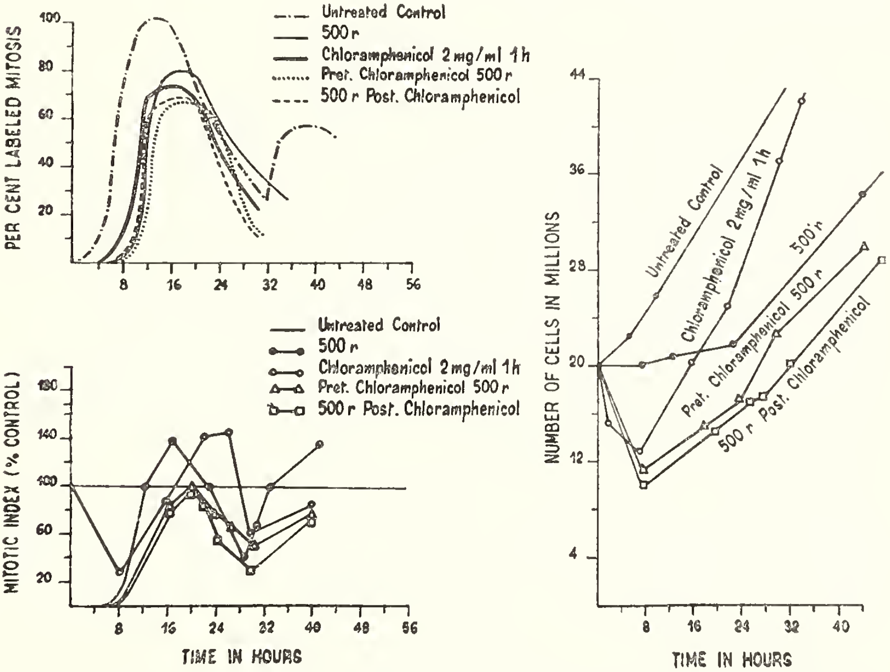
The number of cells decreased during the first 8 hours after irradiation, then grew logarithmically. This loss of cells immediately after the treatment and the fact that the percentage of labeled interphases was similar to the control at 4 hours and increased in the samples fixed after

8 hours are indications that a fraction of the unlabeled cell population died soon after the treatment. This lethal effect might be caused mainly by the inhibition of protein synthesis in those G_2 cells that were prepared for the beginning of mitoses, but it is also possible that the inhibition of protein synthesis is lethal also for G_1 cells.

The surviving fraction of the cell population restored tumor growth, reaching the same number of cells as the untreated 40 hours after the treatment.

EFFECT OF CHLORAMPHENICOL AND X RAYS

X rays combined with pretreatment or posttreatment with chloramphenicol produced the following effects (text-fig. 4).



TEXT-FIGURE 4.—Effects of chloramphenicol 2 mg per ml given alone during 1 hour (O–O), as a pretreatment of 500 r (△–△), or as posttreatment after 500 r (□–□) on: 1) the percent labeled mitoses (*upper left*), 2) the mitotic index (*lower left*), and 3) the number of cells per animal, measured as a function of time after X irradiation. Cells were labeled *in vitro* for 20 minutes before X irradiation, and followed *in vivo* up to fixation.

Mitotic Index

Five hundred r preceded by or followed by chloramphenicol gave a mitotic inhibition similar to that induced by irradiation alone, but the peak of mitotic accumulation reached the control value at 20 hours, decreasing afterward up to 30 hours. The second peak was reached after 40 hours.

Percentage of Mitoses With Labeled DNA

The curve of labeled mitoses seen after 500 r and pretreatment or posttreatment with chloramphenicol was similar to that induced by 500 r alone, although the highest percentage of labeled metaphases was 60 percent.

Chromosome Breakage

Pretreatment with chloramphenicol produced a high increase of the frequency of breaks induced by 500 r in unlabeled and labeled cells, but the maximum effect was seen in the unlabeled cells from G_2.

Posttreatment also increased the number of breaks but the effect was lower than that induced by pretreatment.

Cell Lethality

Inhibition of protein synthesis before or after irradiation with 500 r reduced the number of cells up to the 8th hour. Thereafter, the cell population increased slowly, reaching at 30 hours the same number of cells inoculated at time 0. Since during this period the proportion of labeled interphases increased, it is possible that cell death takes place in part of the cells in G_2 and G_1 during treatment.

When the inhibitors of protein synthesis were given before or after 2000 r, a significant increase in the lethal damage was found (*see* text-fig. 3). The number of cells decreased up to the 8th day, after which tumor development was inhibited in nearly 40 percent of the animals. The maximum effect was obtained when both pretreatment and posttreatment were given with 2000 r: Tumor growth was inhibited in all the animals.

These results indicate that temporary inhibition of protein synthesis itself is lethal to a small fraction of the cells, mainly those in G_2 during the treatment. It is also possible that some G_1 cells were damaged by the treatment. The effect was enhanced by irradition of the cells before or after the inhibition of protein synthesis, and the increased lethality was expressed as the death of the whole cell population when protein synthesis was inhibited before and after irradiation with 2000 r.

EFFECT OF INHIBITORS OF RESPIRATORY METABOLISM

Sodium azide, at a concentration of 0.001 to 0.005 M, is an inhibitor of the respiratory chain phosphorylation whose terminal reaction is the formation of adenosine triphosphate (ATP). It has uncoupling activity, inhibits ATP ^{32}Pi exchange, and has adenosinetriphosphatase activity [Lehninger, 1959 (*25*)]. Since ATP is a source of energy necessary for other synthetic processes and is necessary for rejoining chromosome breaks induced by X rays [Wolff and Luippold (*10, 26*)], it was of interest to know its effects on mitotic delay and lethal damage, as well as on chromosome breakage.

EFFECT OF SODIUM AZIDE ALONE

Treatment for 1 hour with sodium azide (0.005 M) induced the following modifications.

Mitotic Index

An inhibition of mitotic activity was seen in the first hours after treatment, reaching the lowest mitotic index (20% of the control) at 8 hours. Following this depression there was an increase in cell division and a peak of accumulation of mitoses, reaching 200 percent of the control at 16 hours. The mitotic index was over the control values from 12 to 24 hours and below the control (80%) from 24 up to 30 hours.

Percentage of Mitoses With Labeled DNA

A few labeled metaphases were seen in the samples fixed at 8 and 12 hours, but the proportion of labeled mitoses increased slowly reaching 30 percent at 16 hours, which is an indication that mitotic delay occurred in those cells in G_2 and S during treatment. The maximum percent of labeled mitoses was 60 percent and was reached 24 hours after treatment. Since the proportion of labeled interphases was normal at 8 hours and decreased afterward, this low percentage of labeled metaphases could be due to lethal damage to cells in S.

Chromosome Breakage

The treatment with sodium azide alone induced only 0.1 breaks per cell in the samples fixed 24 hours after treatment. This number is so low we may conclude that the treatment alone did not induce chromosome breakage.

Cell Lethality

The death of one third of the cell population was detected at 5 to 6 hours after treatment. Since no modification of the proportions of labeled and unlabeled interphases was observed during the first 8 hours, this decrease in the number of cells observed soon after treatment could be explained by lethal damage to cells treated during G_2 and G_1 as well as during S. After 6 hours, the number of cells increased, reaching, at 20 hours, the same number inoculated at time 0.

During this time, however, the percentage of labeled interphases decreased, which indicated that while the surviving unlabeled cells from G_2 and G_1 were dividing and increasing their proportion in the cell population, the cells treated during S were dying and their proportion was decreasing, reaching values as low as 6 to 10 percent at 28 to 30 hours. This reduction in the proportion of labeled metaphases and interphases is due to lethal damage of the cells synthesizing DNA and cannot be interpreted as an inhibition in the incorporation of the tritiated thymide caused by sodium azide, because the cells were pulse-labeled before the beginning of the sodium azide treatment.

EFFECT OF SODIUM AZIDE AND X RAYS

When the same concentration of sodium azide was administered before or after X irradiation, the effects observed were as follows (text-fig.5).

Mitotic Index

Either pretreatment or posttreatment with sodium azide lengthened the period of mitotic inhibition as compared to that induced by 500 r alone. Few mitoses were seen up to 12 hours. The mitotic index increased slowly up to 16 hours and remained at about 40 percent of the control up to 22 hours. After this, the mitotic activity continued to be low up to 40 hours, when sodium azide had been administered as pretreatment, but reached a peak of 120 percent of the control at 26 hours after posttreatment. In the latter instance, the mitotic index decreased again up to 40 hours, then increased.

Percentage of Mitoses With Labeled DNA

After pretreatment with sodium azide followed by 500 r, the percentage of labeled mitoses increased slowly, reaching a peak of only 30 percent at 20 to 24 hours and decreased again to 10 percent at 30 hours.

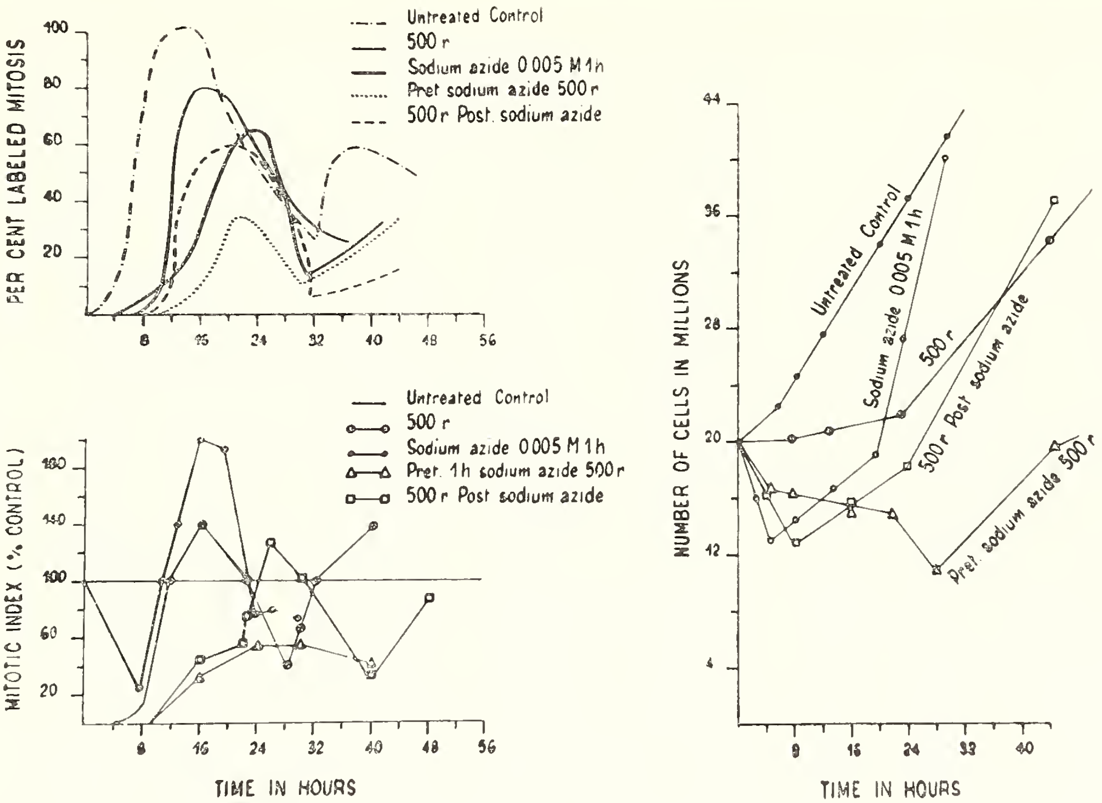
After 500 r and posttreatment with sodium azide the curve of labeled metaphases was similar to that described for 500 r alone, but the highest percentage reached was 60 percent.

Chromosome Breakage

Pretreatment with sodium azide (0.005 M) for 1 hour increased the frequency of breaks induced by 500 r in unlabeled cells from G_2 and G_1 and in labeled cells from S. The maximum effect was observed in the unlabeled G_2 cells that reached metaphase 12 to 16 hours after irradiation and in those labeled cells from early S, which reached metaphase at 24 hours.

Posttreatment did not modify the frequency of breaks induced by 500 r alone.

The increased breakage induced by pretreatment could be due to reduction in the amount of ATP available for restitution. However, the inhibitor given after irradiation might be ineffective due to the presence



Text-figure 5.—Effects of sodium azide 0.005 M given alone during 1 hour (○–○), as a pretreatment of 500 r (△–△), or as a posttreatment after 500 r (□–□) on: 1) the percent labeled mitoses (*upper left*), 2) the mitotic index (*lowerleft*), and 3) the number of cells per animal, measured as a function of time after X irradiation. Cells were labeled *in vitro* for 20 minutes before X irradiation, and followed *in vivo* up to fixation.

of ATP previously formed. Posttreatment with sodium azide might reduce the quantity of ATP only after the X-ray damage had been repaired.

Supposedly, the energy produced by ATP would be necessary for repair of chromosome breaks throughout the mitotic cycle; nevertheless, the maximum effects are seen during G_2 and early S. Perhaps these two periods of the cell cycle require extra energy due to protein synthesis and DNA synthesis, respectively.

Cell Lethality

With either pretreatment or posttreatment, 500 r combined with sodium azide induced a reduction in the number of cells observed up to 8 hours, similar to that produced by the treatment with sodium azide alone.

After that time, the cell population increased if the inhibitor was added as a posttreatment, reaching at 24 hours values similar to those observed after 500 r alone.

With pretreatment, however, a reduction in the number of cells continued up to 28 hours, reaching half of the initial number of cells inoculated at time 0. The division of the surviving cells then restored tumor growth, and after 44 to 48 hours the cell population again reached its original number.

We may conclude that pretreatment with sodium azide produced significant sensitization of the cells and therefore a high increase in lethal damage over that induced by either sodium azide alone or X rays alone. The posttreatment did not produce sensitization and additive effects were found.

When sodium azide was given before a dose of 2000 r, a decrease to half the number of cells was seen at 24 hours (*see* text-fig. 3). It reached one fourth at 48 hours and continued decreasing slowly. Finally, a 100 percent inhibition of tumor development was observed in the animals inoculated with tumor rinsed immediately after irradiation. If the inhibitor was not eliminated after irradiation, the inhibition was found in only 80 percent of the animals. This surprising result might be explained if the presence of sodium azide after irradiation could revert, in part, the sensitizing effect of the pretreatment.

Sodium azide given after 2000 r did not modify the growth curve described for 2000 r. After 4000 r, which is a 100 percent lethal dose by itself, protection of some cells was induced, and tumor growth was recovered (*12*).

DISCUSSION AND CONCLUSIONS

Effect of Inhibitors on Mitotic Delay, Chromosome Breakage, and Cell Lethality in Relation to Differential Radiosensitivity of the Cell Cycle

Variations in cell stage radiosensitivity have been observed in mouse or hamster cell lines, as well as in ascites tumors, when mitotic delay, chromosome breakage, or cell death was observed. That the results described by different authors do not always agree could be explained by the fact that different tissues or cell lines might differ in their radiosensitivity or that different conditions of irradiation were used. Since the results described above may clarify the nature of these differences, it is interesting to discuss the action of X rays and the modifications induced by the inhibitors in relation to cell stage radiosensitivity.

As we have pointed out, each inhibitor produced a different effect on mitotic delay, chromosome breakage, and cell death; thus we consider that the three processes are closely related but do not depend on a common mechanism, and it is appropriate to examine them separately.

Studies on *mitotic delay* show that G_2 is the most sensitive period. A block in this period explains the mitotic inhibition seen immediately after irradiation (*11*, *27*, *28*), and, when the block is overcome, labeled and unlabeled cells reach metaphase.

This delay lasts for 5 to 6 hours after a dose of 500 r, and it is not related to the length of the G_2 period or to the total generation time of the cells studied. For instance, in our ELD hyperdiploid ascites tumor subline, with a generation time of 28 hours and a G_2 of 6 hours, it is similar to that found in the hypotetraploid LP59 mouse fibroblast subline, with a generation time of 22 hours and a G_2 of 2.3 hours (*11*).

After the same dose, the cells from late S reached metaphase after a delay of 5 to 6 hours. These cells could be blocked in G_2 as suggested by Kim and Evans (*27*), or might be blocked during late S and pass through a normal G_2.

Mak and Till (*28*), studying the rate of entry of cells from S to G_2, found a block at the end of S. When this block was overcome, the rate of flow out of S was greater than the control, because cells from different parts of S complete DNA synthesis simultaneously. Moreover, cells in early S were not delayed (*11*, *28*), and the length of the S period was generally not prolonged.

A prolongation of S was found by Kim and Evans (*27*), who incubated the cells at 37° before X irradiation, but since the prolongation is seen only when the cells are pretreated at 37°, their results could be related to the experimental conditions used.

Cells in G_1 were not delayed, and a continuous flow of cells into S was determined [Mak and Till (*28*)]. Nevertheless a reduction in the rate of entrance was found.

The causes for the mitotic block seen at the end of S and G_2 are unknown. Rustand (*29*) postulated that X irradiation could affect the replication or separation of the centriole, and Kim and Evans (*27*) suggested that it could be related to the damage of a nuclear "trigger" for centriole replication.

The idea of a mitotic "trigger mechanism" which makes the initiation of cell division irrevocable was proposed by Mazia (*30*) and Swann (*31*), and the possiblity that such a "trigger" could be related to protein synthesis was discussed by Jacobson *et al.* (*32*). These authors observed that chloramphenicol and X rays produced a similar delay in the division cycle of *Chlamydomonas.*

Our results show that the mitotic delay induced in G_2, when protein synthesis was inhibited for 1 hour, was similar to the delay induced by X rays alone. According to these data, possibly the cause of mitotic delay induced by X rays could be the inhibition of protein synthesis generally, or of some specific enzyme necessary for the beginning of mitosis.

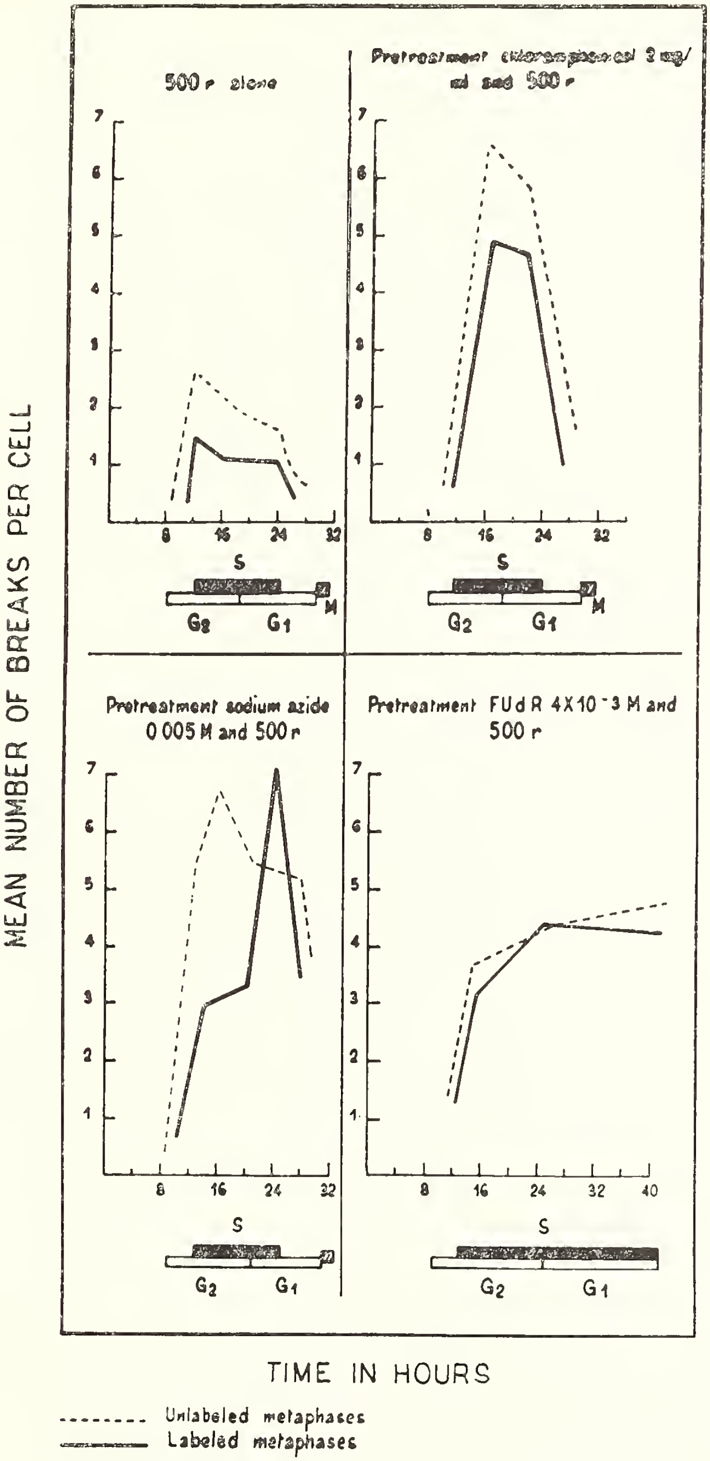
It is well known the differences observed in the frequency of *chromosome breakage* depend on the stage of the mitotic cycle in which the cells are irradiated (text-fig. 6).

An increase in the frequency of aberrations from G_1 to S and from S to G_2 has been observed by Evans and Savage (*33*), Evans and Scott (*34*), and Brewen (*35*) in *Vicia faba.* The same results were obtained by Hsu *et al.* (*36*) and Chu (*37*) in Chinese hamster fibroblast lines. Dewey and Humphrey (*11*), on the other hand, studying a hypotetraploid LP59 mouse fibroblast line and a hyperdiploid Lettre's mouse ascites tumor, found more damage in the labeled anaphases corresponding to cells irradiated during S than in the unlabeled ones in G_2 during the irradiation. Das and Alfert (*38*), studying the proportion of labeled and unlabeled anaphses of *V. faba,* also concluded that the sensitivity to breakage by X rays is at its maximum during DNA synthesis. Probably the differences observed, when aberrations were analyzed, at anaphase are due to difficulties in scoring of many chromatid breaks and chromatid exchanges not leading to the formation of bridges and fragments at anaphase, and not to real differences in sensitivity of the tissues examined. Our results with breaks scored in unlabeled and labeled metaphases of the ELD Ehrlich ascites are in agreement with those described for *V. faba* (*33–35*) and hamster cells (*36, 37*).

Evans and Savage (*33*) and Evans and Scott (*34*) suggested that the increase in sensitivity from G_1 to S could be the result of the doubling of chromosomes during S. Wolff (*39*) considered that it can depend only on the impossibility to score accurately the symmetrical exchanges induced during G_1, whereas the chromatid aberrations produced during S and G_2 are easily scored, whether symmetrical or asymmetrical.

The increase in sensitivity occurring between S and G_2 cannot be explained by the above hypotheses. Brewen, testing the site hypotheses of Wolff and Atwood (*40*), found that the increase in frequency of aberrations seen from S to G_2 was not at random. While the frequency of one-break aberrations remained constant, the two-break aberrations increased

TEXT-FIGURE 6.—Frequency of breaks induced by 500 r alone, or by 500 r after pretreatments for 1 hour with chloramphenicol (2 mg/ml), sodium azide (0.005 M), or FUDR (4×10^{-3} M). Breaks scored in unlabeled metaphases (-----) corresponding to cells irradiated during G_2 or G_1, and in labeled metaphases (———) treated during S. Cells were labeled during the last 20 minutes of the pretreatment and then X-irradiated immediately.


500 r alone
Pretreatment chloramphenicol 2 mg/ml and 500 r
Pretreatment sodium azide 0 005 M and 500 r
Pretreatment FUdR 4X10⁻³ M and 500 r
MEAN NUMBER OF BREAKS PER CELL
7
6
5
4
3
2
1
8
16
24
32
40
S
G2
G1
M
TIME IN HOURS
Unlabeled metaphases
Labeled metaphases

by a factor greater than 4. Since the frequency of the latter is related to metabolic processes necessary for rejoining, it follows that his results could be explained by the varying metabolic requirements necessary for the repair at different stages of the cell cycle. Interestingly, inhibition of protein synthesis increased the frequency of breaks mainly during G_2 and late S, while a reduction in the amount of ATP increased the frequency of breaks all over the mitotic cycle, having its main effect in middle G_2 and early S. Both protein synthesis and the formation of ATP by cell respiration were necessary for restitution during G_1 [Wolff (*41*)], but the intensity of the damage induced at different stages by the same concentration of the inhibitor or by X irradiation could depend on the metabolic requirements of the cells to maintain the integrity of the chromosome at different moments of the cell cycle. For instance, Konrad (*42*) found that the maximum rate of protein synthesis, measured by the incorporation of C^{14} phenylalanine in hamster and human amnion cells, occurred during prophase and falls at metaphase-anaphase, and Baserga (*43*) observed maximal incorporation of C^{14} leucine during the S period in Ehrlich ascites cells. Moreover, Prescott and Bender (*44*), labeling the amoeba nucleus with a mixture of six tritiated amino acids during more than half of the cell cycle, including the entire DNA synthetic period, concluded that nuclear proteins are turning over continuously during interphase, suffer a massive release to the cytoplasm during mitosis, and return to the nucleus at the end of division. They also observed light labeling of metaphase chromosomes with H^3-amino acids and considered that during mitosis the chromosomes contain only small amounts of protein. These findings might explain why periods G_2 and S are the most sensitive to breakage when protein synthesis inhibitors are used and why the sensitization falls abruptly at the beginning of G_1 and during mitosis.

A point of interest regarding the effect of the inhibition of oxidative phosphorylation is the difference observed between the high sensitizing effect of pretreatment and the absence of effect of posttreatment. Apparently, these differences might be related to the existence in the cells of reserves of energy [Mazia (*30*)] that permit restitution during a certain period after the inhibitor is applied. If the inhibitor is given long enough before irradiation, the ATP level drops and less restitution takes place.

A point still difficult to understand is the increased frequency of breaks in unlabeled G_2 or G_1 and labeled cells from S, when FUDR was applied either as pretreatment or posttreatment. FUDR is known to be an inhibitor of DNA synthesis, and the increased breakage observed in the combined treatments with X rays led Taylor (*45*) to postulate that DNA synthesis is necessary for repair of the breaks induced by X rays. Bell and Wolff (*46*) found, as we have, that FUDR may induce breaks in unlabeled chromosomes of G_2, and concluded, therefore, that DNA synthesis is not involved in the production of these breaks. Our results, showing the same increase in the frequency of breaks whether or not the cells were synthesizing DNA at the time of the combined treatment, indicate that another metabolic process, taking place all over the mitotic cycle, could be responsible for the above-mentioned effect. Since FUDR is incorporated into RNA after its cleavage to 5-fluorouracil [Harbers *et al.* (*47*)], an alternative could be that the effect on breakage is related to inhibition of RNA synthesis. This possibility is suggested also by the fact that the addition of thymidine and uridine together with FUDR reduced the frequency of breaks in labeled and unlabeled metaphases.

Lethal damage produced by X rays at different stages of the cell cycle was studied by Terasima and Tolmach (*48*) in a synchronously dividing population of HeLa cells. They found that mitotic cells were maximally sensitive, while cells in early G_1 were relatively resistant. A second maximum sensitive period was found at the end of G_1 or in early S and decreased during DNA synthesis. Cells in G_2 seemed as resistant as those from early G_1. In the same form, Erikson and Szybalski (*49*), studying the cell survival of a human cell line D 98/AG synchronized with FUDR, observed that radioresistance increased during DNA synthesis and decreased when cell division began.

Another example of variation of radiosensitivity through the cell cycle was shown by Whiting *et al.* (*50*) in Habrobracon. They found that metaphase eggs are 22 times more sensitive than prophase eggs, which is difficult to interpret if the main mechanism for cell killing is the damage of DNA [Alper (*51*)].

On the other hand, it was observed, in haploid yeast, that single cells showed 8 times more lethal damage than budding cells, and these two types of cells showed different behavior toward oxygen effect and the damage induced by X rays and α-rays. Alper (*51*), discussing these results, proposed that structures or chemical substances other than DNA could act in part as a target for cell killing.

Our results, showing that a short period of inhibition of protein synthesis or of cell respiration, without irradiation, induces mitotic delay and kills a part of the cell population, but does not break the chromosomes, are good evidence for believing that cell death may be the result of nonchromosomal as well as chromosomal damage. This nonchromosomal damage could be the inactivation of molecules related or not to DNA synthesis. Each one of these targets might be responsible for sensitization at a different period of the cell cycle, since the metabolic requirements necessary for cell survival might be different at different stages.

Cell death caused by inhibition of protein synthesis seems to be induced mainly in G_2 cells and probably also in G_1 cells. The mechanism of death is not clearly understood, but might be explained by the arrival of G_2 cells to a "point of non-return" when they cannot synthesize the necessary proteins to continue through prophase.

On the other hand, lethal damage induced during S by inhibitors of oxidative phosphorylations would be related to a reduction in the amount of ATP available for DNA synthesis, and another type of "point of non-return" would be reached.

The lethal damage induced by FUDR was observed mainly in cells X-irradiated in G_2 and late S when a high concentration of the inhibitor

(4×10^{-3} M) was given as a posttreatment. Here, too, lethality apparently was not due to increased frequency of aberrations, since a high (4×10^{-3} M) concentration of FUDR administered as a posttreatment of 500 r produced the same amount of breaks as pretreatment or posttreatment with a low (4×10^{-5} M) concentration. The treatments with lower concentrations, however, did not increase the lethality, whereas the higher concentrations did. The lethal effect produced by the posttreatment apparently is unrelated to the well-known action of FUDR on thymidilate synthetase or its conversion into 5-fluorouracil, in which form it could be incorporated into RNA, since the effect was reverted neither by thymidine (4×10^{-3} M or 4×10^{-2} M) nor uridine (4×10^{-3} M). It would seem due to some other unknown biochemical action of FUDR on molecules necessary for cell survival. A possible mechanism is suggested by the results of Lindner (*19*). He found a strong inhibition of sulfhydryl groups associated with a high increase of disulfide groups after treatment of ascites tumor cells with 5-FU. As the protein-sulfhydryl groups are necessary for the activity of many enzymes, and sulfhydryl and disulfide groups are important for spindle formation, cell division, and cell metabolism, the increased lethality migh be related to the imbalance in sulfhydryl disulfite ratio.

To conclude, we postulate, on the basis of the evidence presented in this paper, that increased lethality induced by modifications of cell metabolism is not always related to an increase in the frequency of chromosome aberrations, and that lethal damage induced by X rays in a cell population is due only in part to chromosomal damage. Another important part might be due to the inactivation of metabolic pathways related or not to DNA synthesis.

RESUMEN

Las modificaciones inducidas por inhibidores metabólicos, administrados antes o después de la irradiación con rayos X, sobre el retardo mitótico, aberraciones cromosómicas y daño letal fueron estudiadas en una población de células de tumor ascítico de Ehrlich, sublinea ELD.

Se estableció que la duración de una generación celular es de 28 horas; que G_2 tiene una duración de 6 horas, S de 13 a 15 horas, G_1 aproximadamente 6 a 8 horas y la mitosis dura 0,75 horas.

Después de la irradiación con 500 r, se encontró un retardo mitótico de 6 horas, debido principalmente a un bloqueo en G_2. El tratamiento con FUDR solo, prolongó el tiempo de S. El tratamiento combinado de FUDR más 500 r, indujo un bloqueo en G_2 y una prolongación de S. Los tratamientos ya sea con cloramfenicol ó azida sódica solos, retardaron las célulus en G_2, pero no aumentaron en forma significativa el retardo inducido por rayos X solos.

La frecuencia de roturas cromosómicas inducidas por 500 r aumentó con pre ó postratamientos con los inhibidores, según el período del ciclo celular en que se encontraban las células durante el tratamiento. Por ejemplo, el pretratamiento con azida sódica incrementó la frecuencia de roturas en todos los períodos del ciclo mitótico, pero produjo un efecto máximo en G_2 medio y S temprano.

El pretratamiento con cloramfenicol aumentó el número de roturas, principalmente

en G_2 y S tardío, lo que indicaría que el requerimiento de síntesis de proteínas necesarias para reparar el daño cromosómico, aumenta durante esos períodos. Los pretratamientos o postratamientos con FUDR aumentaron las roturas tanto en células marcadas como no marcadas de G_2, S y G_1, lo que señala que dicho efecto está relacionado con algún otro proceso metabólico, y no con la síntesis de ADN.

El daño letal también fué incrementado por algunos inhibidores, pero el período del ciclo celular en que este efecto fué máximo, no siempre coincidió con el de mayor sensibilidad para roturas cromosómicas. Con una dosis de 500 r, ningún inhibidor modificó el porcentage de tomas del tumor, pero se observó una reducción del número de células, durante las primeras horas. Con esa dosis de rayos X, el pretratamiento con azida sódica mató principalmente a células de S, el de cloramfenicol dañó las células de G_2 y tal vez algunas de G_1, y los postratamientos con FUDR resultaron letales para muchas células de G_2 y S.

Cuando estos inhibidores se emplearon junto con 2000 r, el pretramineto con azida sódica y el pretratamiento y postratamiento con cloramfenicol resultaron letales para toda la población celular. FUDR administrado después de la irradiación, en concentraciones elevadas, dañó un alto porcentaje de la población celular y el crecimiento tumoral fué inhibido en 80% de los animales inoculados. Este último efecto no fué revertido por tratamientos simultáneos con timidina ó uridina, por lo que no parece ser debido a inhibición de la síntesis de ADN ó ARN.

Estos hallazgos nos han llevado a postular que el retardo mitótico, las roturas cromosómicas y la muerte celular son tres fenómenos independientes, no producidos por un mecanismo común. Consideramos además, que el daño letal inducido en una población celular es solo debido en parte a roturas cromosómicas y que otra parte importante de dicho daño es causado por la inactivación de ciertas moléculas, relacionadas o no con la síntesis de ADN.

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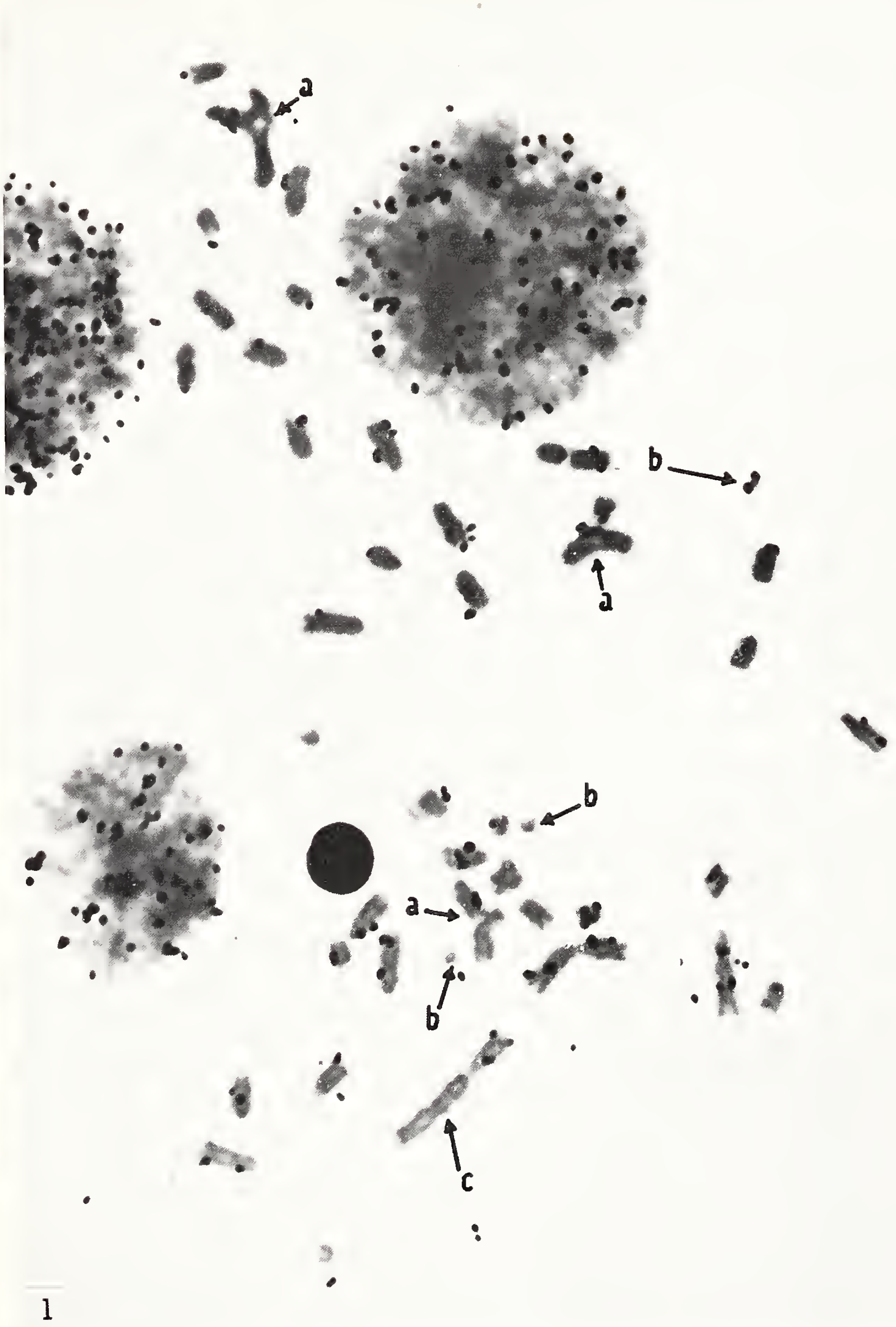
Plate 24

Figure 1.—An autoradiograph of ELD Ehrlich ascites cells incubated for 20 minutes with H^3-thymidine, immediately before X irradiation with 500 r. Nuclei of the interphase cells and the chromosomes of a metaphase are labeled. *Note* the type of chromosome aberrations: (*a*) chromatid exchanges, (*b*) isochromatid breaks, and (*c*) a chromosome exchange.



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DISCUSSION

Evans: Apart from one study on mouse cells by Dewey and Humphrey,* all observations on aberrations induced by ionizing radiations in mammalian cells indicate that cells in G_2 may be more sensitive than cells irradiated in other stages of the cell cycle. In Dewey and Humphrey's studies on mouse cells, in which G_2 was claimed to be a less sensitive phase than S, the doses employed resulted in a complete absence of mitosis for a 6-hour period which corresponded with that time when G_2 cells should be entering division. Since these data on mouse cells vary from all the other mammalian data and, indeed, other plant data, I believe that in the mouse experiments the doses used were too high, such that they killed off G_2 cells in interphase so that these cells, which may be the most sensitive to aberration induction, never appeared at mitosis and could not be scored. I should like to ask Dr. de Lozzio and Dr. Valencia whether they observed any interphase cell killing with the doses used in their experiment, and, if such killing was observed, to what extent are the aberration frequency results distorted by this imposed cell selection.

de Lozzio: When a metabolic inhibitor was used before or after irradiation with 500 r, we observed a decrease in the number of cells during the first 8 hours following irradiation. This is the only indication we have of the possibility that cell death occurs before metaphase, but it may represent death of the most sensitive cells. Therefore, when we later scored the frequency of chromosome aberrations, we were studying the chromosomal damage induced in the fraction of surviving cells instead of analyzing the whole irradiated cell population. However, 500 r alone did not reduce the number of cells and the inhibitors alone resulted in a reduction in the number of cells. But since no chromosome aberrations were detected, we believe the cause of cell lethality immediately after irradiation would be the inhibition of metabolic processes necessary for cell division, instead of increased chromosomal damage. For that reason, we consider the frequency of breaks scored at different times of fixation is a good measure of the chromosomal damage, though we could not determine the number of aberrations induced in the dead cells.

Concerning the observations of Dewey and Humphrey we would like to add some comments. We think their results are due to the fact that they scored anaphases instead of metaphases, and therefore, they could not detect many chromatid breaks and chromatid exchanges induced during G_2. A similar pattern of sensitivity to breakage was found by Das and Alfert†, scoring anaphases in *Vicia faba*. Nevertheless, all the studies made in *Vicia faba* analyzing metaphases have shown a higher frequency of breaks in cells irradiated during G_2. Also, the complete absence of mitoses, observed by Dewey and Humphrey and by us during the 6 hours following irradiation, does not represent cell killing of G_2 cells, but mitotic delay during this phase of the cell cycle. This is indicated by the accumulation of mitoses seen from 12 to 24 hours after irradiation, with a peak at 16 hours. At this time 20 percent of the cells reaching metaphase were unlabeled, whereas the rest (80%) were labeled. The arrival at metaphase of the delayed G_2 cells, together with the nondelayed S cells, does not permit the detection of differences in sensitivity of cells from G_2 and S, if the frequency of breaks is not scored separately in unlabeled and labeled cells fixed at 12 and 16 hours after irradiation. In doing so, we found a higher frequency of breaks in the unlabeled G_2 cells than in the labeled cells from S, therefore concluding that in the mouse, as well as in *Vicia faba*, or hamster cells, the most sensitive period for chromosome breakage is G_2.

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Chromosome Behavior

Chromosome Pairing, Crossing-Over, and Segregation in *Drosophila Melanogaster* [1,2]

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SUMMARY

The following points briefly recapitulate recent findings concerning *Drosophila* oogenesis: 1. Nonrandom assortment of nonhomologous chromosomes has been demonstrated for all of the chromosomes of the *Drosophila* genome. The equality of reciprocal classes from such assortments indicates that they are the result of meiotic segregations in which associations between nonhomologues precede segregation of nonhomologues to opposite poles. Segregation of nonhomologues from one another may be as regular or more regular than segregation of homologues. 2. Crossing-over studies have shown that chromosomes participating in nonhomologous associations are always noncrossovers. They have also shown that the incidence of such events, involving one of a pair of homologues does not affect the frequency of exchange between the two homologues. These two findings have led to a new model for meiosis. This model includes two types of pairing, an early type, concerned with exchange, called *exchange pairing*, and a later type, concerned with segregation, called *distributive pairing*. 3. Investigations into the nature of distributive pairing have disclosed that it is a size-dependent process such that both pairing frequencies and pairing preferences displayed at this time correlate extremely well with similarity in mitotic metaphase lengths of the participating chromosomes. In this respect it differs from exchange pairing, where the prime concern is homology. The dependence on total lengths excludes the possibility that association between nonhomologues at the distributive phase is a consequence of shared homology in proximal heterochromatic regions. 4. Distributive pairing has been shown to be the mechanism responsible for regular segregation of the fourth chromosomes under normal conditions. 5. The distributive pairing theory provides a satisfactory explanation for a number of heretofore unexplained situations, including regular segregation without exchange, nonhomologous pairing, the mechanism of secondary nondisjunction, and preferential segregation of the fourth chromosomes. 6. Evidence bearing on the coincidence of exchange and DNA replication is presented. It is suggested that parasynapsis of homologues prior to prophase, followed by exchange, concurrent with or close to the time of DNA replication, and distributive pairing at late prophase or metaphase presently provide the most satisfactory correspondence between genetic events and the cytological picture.—Nat Cancer Inst Monogr 18: 215–242, 1965.

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

THE STUDY of chromosome behavior during meiosis no longer occupies the preeminent position that it did in the early days of genetic investigation. This does not mean that all or even most of the problems of chromosome transmission have been solved. Among many organisms customarily used for genetic studies, such as *Neurospora*, maize, and the mouse, transmission genetics is in a fairly elementary state. Even *Drosophila*, where such studies have been most extensively pursued and where the mechanisms were considered to be fairly well understood, has proved to be a still rewarding organism for investigation. Here, some exceptional findings which have led to a radical revision in our concept of the meiotic mechanism have recently come to light. It is about these findings and their implications that this paper is concerned.

CLASSICAL MODELS OF MEIOSIS

We will begin with the premise that the basic processes we are dealing with during meiosis are chromosome pairing, chromosome replication, chromosome exchange, and chromosome disjunction. Our need is to fit these events into a causal relationship consistent with our fund of genetic, biochemical, and cytological information. Superficially, this would appear to be a simple task because the possibilities are few and because they must be restricted to accommodate the following facts: 1) that some type of pairing must precede crossing-over; 2) that crossing-over in the four-strand stage probably means that chromosome replication also precedes or is concurrent with exchange; 3) that crossing-over must occur before disjunction; and 4) that disjunction itself implies a previous association of chromosomes.

The scheme that was enthusiastically adopted by many geneticists in the thirties was Darlington's "precocity theory" of meiosis (*1*), which took as its basic tenet that homologues pair at zygotene because they enter prophase precociously in an unreplicated state, in contrast to mitotic chromosomes, which are visibly double at the beginning of prophase. The cytological observations of split chomosomes before zygotene (*2*), as well as the recent finding that DNA replication occurs before zygotene (*3*, *4*), no longer permit serious consideration of this hypothesis. From the purely genetic viewpoint, we are concerned with that aspect of Darlington's theory which holds that the presence of a chiasma is a prerequisite for the regular segregation of homologues. According to this model, the casual sequence of events is chromosome pairing, followed by chromosome exchange leading to regular disjunction. Darlington's scheme assumes that chiasmata are generally formed in all paired chromosomes at pachytene and that failure to form a chiasma results in a univalent at metaphase and some nondisjunction or loss at anaphase (*1*).

From the time of its inception, it was apparent that this model was incompatible with certain genetic data. Even if we ignore the male of *Drosophila*, in which regular segregation occurs in the absence of exchange, and limit our considerations to the female, we find that here, too, exchange is not a prerequisite for regular disjunction. Evidence for this comes from the small fourth chromosomes that are invariably noncrossovers in the diploid female but which segregate in a highly regular fashion; from the larger X chromosomes which by tetrad analysis are found to be noncrossovers about 5 percent of the time, but which nondisjoin with only about one hundredth of this frequency, or 0.05 percent (*5*); from those cases where noncrossover X tetrads occur with exceedingly high frequencies due to the presence of heterozygous X inversions, yet where nondisjunction remains less than 1 percent (*6*); and from studies with the mutant c3G which practically eliminates meiotic exchange but where chromosome assortment shows a marked departure from randomness (*7*). As emphasized by a number of workers (*8*, *9*), these observations indicate that something other than exchange is capable of governing or modifying disjunctive behavior.

As an alternative to Darlington's "chiasma hypothesis of metaphase pairing," Dobzhnasky, on the basis of the behavior of translocation heterozygotes, formulated the hypothesis of competitive pairing (*8*). According to this view, crossing-over and nondisjunction are negatively correlated. The relation, however, is not a direct causal one in the sense that crossing-over inevitably leads to regular disjunction, and failure of crossing-over leads to random assortment. Rather, Dobzhansky assumed that both processes are predetermined by the intimacy of synapsis between specific homologous loci, prior to crossing-over. Rearrangements would provoke a conflict between the attraction forces, weaken the intimacy, as well as decrease the frequency of synapsis between homologues, and lead to both decreased crossing-over and increased nondisjunction. This hypothesis could account for the high frequencies of nondisjunction in translocation heterozygotes, despite fairly normal cross-over values, if intimacy of pairing were considered to be a more critical requirement for the disjunctive process than it is for exchange. From inversion heterozygotes, however, where crossing-over is known to be greatly disturbed, disjunction would be expected to be even more irregular. Instead we find it is highly regular, and it becomes apparent that the solution to the problem must be sought elsewhere.

THE PHENOMENON OF NONHOMOLOGOUS PAIRING

This is the way matters stood until the mid-fifties. At this time a remarkable phenomenon was discovered in the *Drosophila* female, which, as it turned out, held the key to many of the principles underlying the behavior of chromosomes during oogenesis. It was found that under the proper conditions nonhomologous chromosomes would segregate from one another with great regularity, and it has been assumed here, as with homologues, that regular segregation is preceded by some type of regular pairing.

That associations between nonhomologues might be occurring was first postulated by Cooper and his co-workers *(10)*, in 1955, to explain the increase both in X chromosome nondisjunction and in dominant lethality when heterozygous X and autosomal inversions were simultaneously present in the genome. Following this, in 1956, Sandler and Novitski *(11)* proposed that pairing between nonhomologues could account for the failure to recover the different classes of progeny from triploids in the frequencies expected from independent assortment. The following year we were able to provide convincing genetic evidence for associations between nonhomologues by studying the offspring of females carrying an extra Y chromosome and a single free fourth chromosome *(12)*. It was found that in over 80 percent of the progeny, if the extra Y was present, the four was not; or conversely, if the four was present, the Y was not. More important, we could establish that the frequency of eggs carrying the maternal free four and lacking the extra Y was equivalent to the frequency of eggs carrying the extra Y and no maternal free four *(12, 13)*. In other words, these two kinds of eggs represented the two reciprocal products resulting from the pairing and subsequent segregation of the two nonhomologues, the Y and the fourth chromosome. Clearly, this constitutes a complete contradiction to the law of independent assortment of nonhomologous chromosomes.

Since then, the combined work of a number of geneticists has disclosed not only that all of the chromosomes of the *Drosophila* genome are capable of participating nonhomologous associations, but that such associations can occur with the utmost regularity, reaching in one case a frequency of 99.9 percent *(14)*. Table 1 provides an indication of the generality and the frequencies of this phenomenon.

While it was of great interest to discover that chromosomes could behave in this unorthodox manner, at the same time this phenomenon presented a serious paradox. All pertinent evidence indicates that crossing-over is an extremely precise process, calling for exact pairing at the nucleotide level to ensure that the DNA template is correctly conserved. Furthermore, it is well known that nonhomologues do not undergo exchange. How then can the prerequisite for completely specific pairing before exchange be reconciled with high frequencies of nonhomologous pairing at this time? Even if it were conceded that chromosomes might conceivably resort to illegitimate pairing in the absence of their homologue, as, for instance, in haploid strains of plants, the paradox is heightened in those cases where both homologues are present, although one may carry a partially inverted sequence, yet a nonhomologue, possessing an entirely dissimilar nucleotide sequence, is apparently preferred.

Sandler and Novitski *(11)* had proposed, as a possible solution, that all chromosomes of the *Drosophila* genome possess regions of shared homology in their proximal heterochromatin. Viewed in this way, nonhomologous pairing becomes entirely a matter of proximal heterochromatic pairing. We were able to test this hypothesis by studying the associations among three chromosomes, two of which carried the same centromere and hetero-

TABLE 1.—Frequencies of nonrandom assortment of different pairs of nonhomologues in *Drosophila melanogaster*

Genotype	Chromosomes involved	Segregation (%)	Reference
$Y;\ \dfrac{T(3;4)\ 86D}{\dfrac{T(3;4)\ 86D}{4}}$	$Y \longleftrightarrow 4$	92	R. F. Grell (*13*)
$Y;\ \dfrac{Ins(2L + 2R)\ Cy}{T(2;3)\ rn}$	$Y \longleftrightarrow 2$	90. 1	T. Oksala (*15*)
$Y;\ \dfrac{Ins\ (3LR)}{T(2;3)\ A}$	$Y \longleftrightarrow 3$	84	B. Miller and R. F. Grell (*16*)
$X\ Dp;\ \dfrac{T(3;4)\ 86D}{\dfrac{T(3;4)\ 86D}{4}}$	$X\ Dp \longleftrightarrow 4$	99. 9	R. F. Grell (*14*)
$\overline{XX}^*;\ \dfrac{SMI,\ Cy}{T(2;3)\ A}$	$\overline{XX} \longleftrightarrow 2$	90	E. H. Grell (*17*)
$Y;\ \overline{44}^*$	$Y \longleftrightarrow \overline{44}$	98. 8	E. H. Grell (*17*)
$\dfrac{SMI,\ Cy}{T(2;3)\ A};\ \overline{44}$	$2 \longleftrightarrow \overline{44}$	95	E. H. Grell (*17*)
$\overline{XX};\ \overline{44}$	$\overline{XX} \longleftrightarrow \overline{44}$	97	E. H. Grell (*17*)

*Symbols: $\overline{XX}$ = compound X; $\overline{44}$ = compound 4.

chromatic base but euchromatic segments of different origin and length, while the third chromosome possessed no known homology for either of the other two (*18*). If nonhomologous pairing is entirely a proximal heterochromatic affair, associations should occur at least as frequently between the two elements with identical bases as between either of these and the third chromosome. Instead, it was found that the two chromosomes with identical bases assorted randomly and that associations occurred exclusively between one of these two and the third chromosome. Apparently then, factors other than the centromere and proximal heterochromatin are involved in nonhomologous associations. Results, to be described later, have now made it abundantly clear that proximal heterochromatin, *per se*, is inconsequential in this process.

TESTS OF THE SINGLE PAIRING MODEL

1. Nonhomologous Pairing

As alternatives to the Sandler-Novitski hypothesis, it may be proposed that nonhomologous pairing occurs either along the entire length of the chromosome or solely in the euchromatic segments. In either case, if such pairing precedes exchange, two consequences are inevitable. First, chromosomes that have been associated nonhomologously must be noncrossovers, and second, crossing-over between homologues must be reduced to the extent that nonhomologous associations occur. Both of these predictions may be tested genetically, and such tests were performed (*19*).

For this test females were constructed that carried a particular chromosome for which a choice in pairing existed. It might pair and perhaps cross over with its homologue, or it might pair with a nonhomologue. Preliminary studies had already shown that in such females this chromosome paired with the nonhomologue about 50 percent of the time. Sister females, identical in every respect to the test females except that they lacked the nonhomologue, were used as controls. According to the single pairing, *i.e.*, classical, hypothesis, nonhomologous associations occurring about 50 percent of the time must lead to a reduction in crossing-over of about 50 percent. Moreover, those chromosomes engaging in the nonhomologous associations are expected to be noncrossovers. Analysis of the results revealed that those chromosomes that segregated from the nonhomologue were indeed noncrossovers, but contrary to prediction, crossing-over was the same whether the nonhomologue was absent or present. The test was repeated with two other genetic situations, but the results were the same in each case. The data, therefore, tell us: 1) that exchange between homologues is not altered by the presence of the nonhomologue; 2) that only those chromosomes which have not participated in exchange with a homologue are available for associations with a nonhomologue. Thus, it becomes clear that nonhomologous associations do not produce noncrossovers, but rather that noncrossovers associate nonhomologously after exchange has occurred.

2. Secondary Nondisjunction

A situation closely analogous to nonhomologous pairing is that which gives rise to secondary nondisjunction. This process has been extensively studied over the years, beginning with the classical work of Bridges (*20*), but it has remained something of a puzzle leading Sturtevant and Beadle (*21*) to remark that "the general problem of the mechanism of secondary nondisjunction is unsolved."

Secondary nondisjunction may be defined as the increased frequency of X nondisjunction that occurs in females bearing a Y chromosome. The consequences of X nondisjunction are recognizable among their progeny either as females possessing both maternal X's or as males possessing neither; both types are collectively referred to as secondary exceptions. Since the nondisjoining X's, in contrast to most of the regularly disjoining X's, are noncrossovers, Bridges postulated that the difference between the two types was initiated at a stage preceding crossing-over, and that the exceptions arose from those cases in which a competition for pairing partners among the two X's and the Y led to an XY bivalent and an X univalent. Secondary exceptions would result when the single X happened to pass to the same pole as the segregating X.

This scheme introduces the same problem that was encountered with nonhomologous pairing, namely, if pairing for exchange is based on homology, and there is every reason to believe that it is, why should an X find the Y, with which it has only one known locus in common, more attractive than its own homologue. Fortunately, the Bridges' model is subject to the same kind of test that was applied to nonhomologous pairing. Bridges' model likewise assumes a single pre-exchange pairing, so that the incidence of XY bivalents must lead to a corresponding decrease in XX bivalents and a concomitant decrease in X exchange. Bridges had, in fact, looked for such a decrease in crossing-over and had failed to find it. Since the genetic backgrounds of the two types of females (XX and XXY) that he used were uncontrolled, his test could not be considered a critical one.

In our tests, sister females were utilized, half of which were XX in genotype and the other half XXY, and the frequencies of X nondisjunction and X crossing-over were measured for the two groups. Studies were made on females that carried two isosequential X's, as well as on females that carried a variety of different heterozygous X inversions (*5*). The results demonstrated that in all cases the presence of a Y in the mother causes an approximately 50-fold increase in X nondisjunction, and, as is well recognized, these secondary exceptions are noncrossovers. Crossing-over studies with two normal X's, however, showed that a Y caused an increase rather than the predicted decrease in crossing-over. With inversion heterozygotes the Y caused localized effects on crossing-over that could be traced to the position of the inversion. In some cases these effects resulted in an over-all increase, in others a decrease, but in the latter case the alteration in crossing-over was never as great as that predicted from the Bridges' model.

That the Y was not producing the noncrossover X's recovered as exceptions was most clearly shown with the normal X's where we could perform a tetrad analysis to determine the number of noncrossover X bivalents. These results are given in table 2. In series 1, the frequency

TABLE 2.—Tetrad analysis of crossing-over in normal X chromosomes

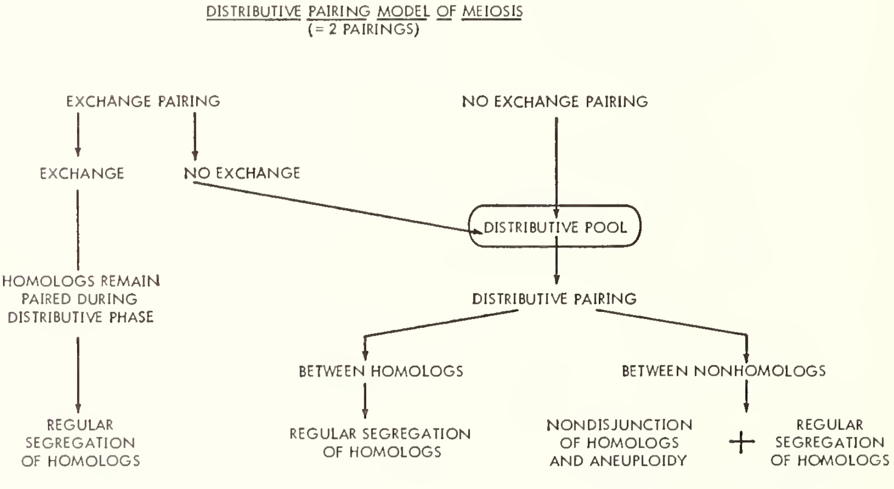
Experiment	E_0*	E_1†	E_2‡	E_3§	Distance measured	Percent total crossing-over
1 XX	4. 73	57. 62	36. 00	1. 64	Distal tip (*sc*) to centromere	67. 24 ± 0. 95
1 XXY	4. 98	51. 77	41. 34	1. 91		70. 09 ± 1. 00
2 XX	5. 23	62. 91	30. 94	0. 91	Distal tip (*y*) to car	63. 76 ± 1. 14
2 XXY	3. 67	60. 73	34. 61	0. 98		66. 44 ± 0. 95

*No-exchange tetrad.
†Single-exchange tetrad.
‡Double-exchange tetrad.
§Triple-exchange tetrad.

of E_0's, *i.e.*, noncrossover X's, is the same for the XX and XXY sisters; in series 2, the E_0's are actually fewer in the presence of the Y, although probably not significantly so. This is so despite a 40-fold increase in X nondisjunction with the Y. The over-all increase in X exchange observed with a Y is attributable to the increase in higher rank tetrads, principally E_2's, at the expense of E_1's. These results demonstrate that the effect of the Y on X disjunction is independent of its effect on X exchange. Furthermore, it is evident that the role of the Y is not to increase X E_0's by pairing competitively with the X's before exchange, but instead to pair with X E_0's *after* exchange and in this way to cause them to nondisjoin.

THE DISTRIBUTIVE PAIRING MODEL OF MEIOSIS

These studies have led to only one harmonious interpretation of the meiotic events in *Drosophila* females. Nonhomologous associations do not occur prior to exchange. Rather, they occur after exchange and exclusively between chromosomes that have failed to undergo exchange. On the basis of this evidence, the model shown in text-figure 1 has been proposed.


DISTRIBUTIVE PAIRING MODEL OF MEIOSIS
(= 2 PAIRINGS)
EXCHANGE PAIRING
NO EXCHANGE PAIRING
EXCHANGE
NO EXCHANGE
DISTRIBUTIVE POOL
HOMOLOGS REMAIN PAIRED DURING DISTRIBUTIVE PHASE
DISTRIBUTIVE PAIRING
BETWEEN HOMOLOGS
BETWEEN NONHOMOLOGS
REGULAR SEGREGATION OF HOMOLOGS
REGULAR SEGREGATION OF HOMOLOGS
NONDISJUNCTION OF HOMOLOGS AND ANEUPLOIDY
+
REGULAR SEGREGATION OF HOMOLOGS

TEXT-FIGURE 1.—Distributive pairing model of meiosis (= 2 pairings).

Chromosomes that share homology with one another pair before exchange. This pairing is called *exchange pairing.* If one chromosome has no homology for another, such as perhaps a Y deleted of its short arm, it will not become involved in exchange pairing. Following such pairing, exchange may or may not occur. When exchange does occur, a chiasma ties the participating chromosomes together, and this tie precludes their subsequent involvement with other chromosomes so that they remain associated during the following stages and segregate regularly from one another. If exchange fails to occur, such chromosomes form a pool of nonexchange chromosomes called the *distributive pool.* Within this pool a second type of pairing, which we call *distributive pairing,* now takes place. Distributive pairing may occur between two homologues, in which case segregation is again regular. Alternatively, it may occur between two nonhomologues, and when this happens the subsequent segregation of the nonhomologues will lead to their nonrandom assortment and part of the time will result in nondisjunction of homologues and in aneuploid gametes.

A clarification of what is meant by a chromosome in the distributive sense is in order. Just as it is normally defined, a chromosome is considered to possess a centromere to which some segment of chromatin is attached. Its form may be metacentric as with the large autosomes, J-shaped as with the Y, or acrocentric as with the X. It may be an element deleted of most of its chromatic material such as a small X duplication, a duplicated element such as a compound X which carries two X's attached to one centromere, or an element composed of portions of different chromosomes such as a part of a reciprocal translocation. All of these types function as single chromosomes. In the case of the compound chromosome, it has been shown by E. H. Grell (*17*) that crossing-over between its two homologous arms does not alter its functional role as a single chromosome, so that a compound X, whether a crossover or a noncrossover within itself, is equally available for distributive pairing.

With this model of meiosis it is now possible to resolve those problems that appeared insoluble with the single-pairing schemes. First, from Darlington's hypothesis we see that crossing-over leading to regular segregation is correct, because crossover bivalents remain associated, do not become involved with nonhomologues, and segregate regularly at anaphase. Does lack of crossing-over necessarily lead to nondisjunction as Darlington assumed? We see it need not, at least in *Drosophila,* for chromosomes that have failed to cross over are afforded a *second opportunity* for associating with one another at distributive pairing. It is this latter type of pairing that is responsible for the regular segregation of X-inversion heterozygotes, despite the fact that the majority may be noncrossovers. Their segregation is regular, however, only so long as a third, suitable chromosome is not present in the distributive pool. Should a Y, for instance, be added, association between the X's and Y will result in high frequencies of X nondisjunction, *i.e.,* the process described as secondary nondisjunction. The more efficient the inversion system in producing noncrossover X tetrads, the more frequently the X's will be members of the pool and the more secondary exceptions will occur. Thus it becomes clear why the Y associates only with noncrossover X's but does not increase their number. Finally, we see that the requirement for precise pre-exchange pairing is maintained, since associations between nonhomologues occur only after exchange at the distributive phase.

CHARACTERIZATION OF EXCHANGE AND DISTRIBUTIVE PAIRING

1. Exchange Pairing

Exchange pairing is defined as occurring exclusively between specific homologous loci; on the molecular level this is equivalent to pairing between identical (or complementary) sequences of nucleotides. If more than two such loci are present, exchange pairing is competitive, since pairing at any particular level is assumed to be limited to two chromosomes. Asymmetrical pairing and exchange, leading to duplicated and deleted chromosome segments, as described for the *zeste* and *white* regions (*22*) or within the *white* region (*23*), are interpreted to be a consequence of duplicated segments which still possess sequences of nucleotides homologous to those of the original segment. This means, as pointed out by Green (*24*), that the terms "nonhomologous pairing" and "nonhomologous exchange" are inappropriate to describe these processes.

2. Distributive Pairing

While exchange pairing is characterized as being extremely precise and limited to homologous regions, distributive pairing, by contrast, may occur as regularly between nonhomologues as between homologues. When more than two chromosomes are present in the distributive pool, it has been shown that pairing is competitive and that marked preferences are displayed (*18*). Preference at distributive pairing is apparently not attributable to homology, since studies with three nonrecombinant chromosomes, described earlier, have shown that those two which segregated regularly shared no known homology, whereas the two with the same basal heterochromatin assorted randomly. The question to be answered, of course, is: What factor or factors are responsible for preferences in distributive pairing? In the experiment just referred to, one obvious possibility was chromosome size, since the two chromosomes that segregated regularly were large, whereas the third chromosome that assorted randomly was small. The series of investigations that will now be described examined the role of size in distributive pairing (*14*).

a. Noncompetitive System

The first set of experiments studied the effect of size on the frequency of distributive pairing between two chromosomes in a noncompetitive situation. One of the two chromosomes was kept constant, while the other was varied from much smaller to much larger than the first. For the variable element, a series of free X-chromosome duplications were used that had been obtained by Cooper and Krivshenko and which had been measured by these workers at mitotic metaphase, using the length of the fourth chromosome as a standard. With respect to chromosome four, arbitrarily given a length of 1, the duplications vary from ≤ 0.3

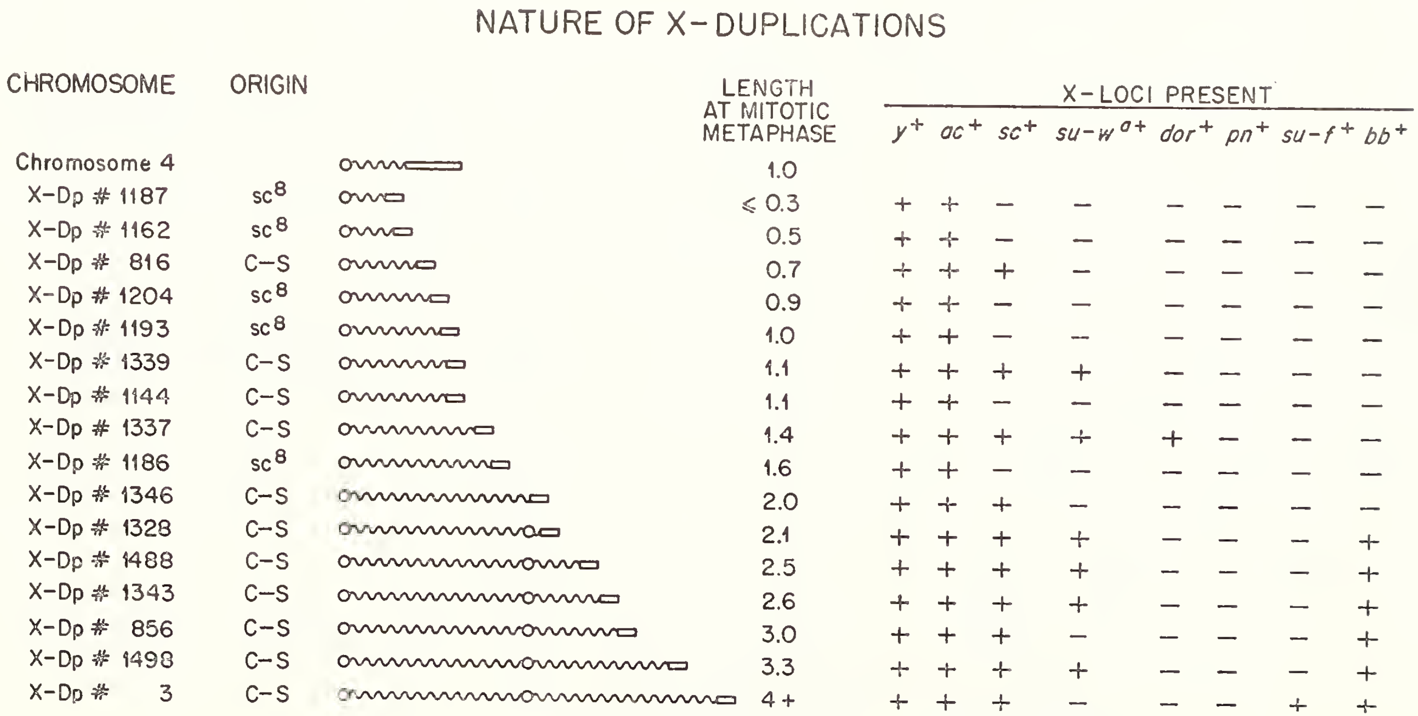
(Dp 1187) to 3.3 (Dp 1498) times the length of chromosome four as shown in text-figure 2. The largest duplication, #3, which is greater than 4 times the length of chromosome four, was obtained by Lindsley and Sandler (*25*).

Each X duplication was introduced into females of identical genotype that carried a single free fourth chromosome which served as the nonvariable element. In this situation, both the free X duplication and the single free four are virtually always noncrossovers and are generally the only two members of the distributive pool. A measure of the frequency of pairing between them can be obtained either from the frequency with which they segregate from each other or from the frequency with which they nondisjoin. Since the frequency of nondisjunction is a measure of the frequency that the duplication and four fail to pair and segregate, the lower the nondisjunction frequency, the greater the frequency of pairing. Table 3 gives the frequencies of nondisjunction between each duplication and the four. The highest nondisjunction frequency, 4.5 percent, occurs with the smallest duplication. As the duplications become larger and approach the four in size, nondisjunction decreases—meaning that association increases. The lowest nondisjunction, and hence the highest association, occurs with those duplications closest to the four in length. For Dp 1144 with a length of 1.1, it is 0.02 percent, or one nondisjunctional product per 5,000 flies. Then, as the duplications become larger than the four, nondisjunction again increases, leveling off at 2 percent for duplications 2.5 or larger. It is evident that the amount of pairing between two nonhomologues, in a noncompetitive situation, is a function of their similarity in size.

TABLE 3.—Noncompetitive system: nondisjunction frequencies between chromosome four and X duplications of variable lengths

Duplication No.	Mitotic length	Totals	Dp, 4 nondisjunction among X regulars* (%)
1187	≤0.3	12,113	4.49 ± 0.19
1162	0.5	8,000	0.39 ± 0.07
816	0.7	9,324	0.22 ± 0.05
1204	0.9	7,391	0.08 ± 0.03
1193	1.0	5,371	0.13 ± 0.05
1339	1.1	7,126	0.08 ± 0.03
1144	1.1	8,476	0.02 ± 0.02
1337	1.4	5,635	0.38 ± 0.08
1186	1.6	5,991	0.39 ± 0.08
1346	2.0	8,011	0.42 ± 0.07
1328	2.1	2,789	0.43 ± 0.12
1488	2.5	2,665	1.94 ± 0.27
1343	2.6	3,343	2.20 ± 0.25
856	3.0	3,732	1.90 ± 0.24
1498	3.3	2,767	1.63 ± 0.24
3	4+	344	2.00 ± 0.75

*X nondisjunction less than 2 percent in all cases.



NATURE OF X-DUPLICATIONS

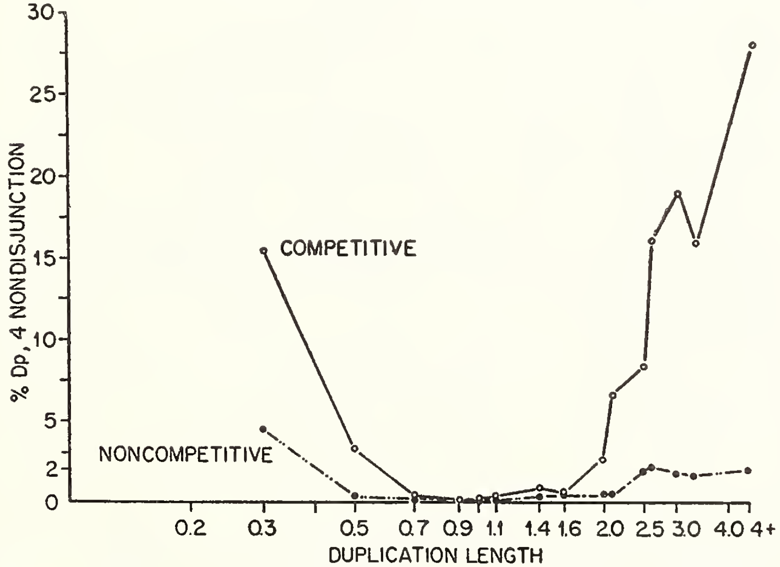
CHROMOSOME	ORIGIN	LENGTH AT MITOTIC METAPHASE	X-LOCI PRESENT y^+	ac^+	sc^+	$su\text{-}w^{a+}$	dor^+	pn^+	$su\text{-}f^+$	bb^+
Chromosome 4		1.0								
X-Dp # 1187	sc^8	$\leqslant 0.3$	+	+	−	−	−	−	−	−
X-Dp # 1162	sc^8	0.5	+	+	−	−	−	−	−	−
X-Dp # 816	C-S	0.7	+	+	+	−	−	−	−	−
X-Dp # 1204	sc^8	0.9	+	+	−	−	−	−	−	−
X-Dp # 1193	sc^8	1.0	+	+	−	−	−	−	−	−
X-Dp # 1339	C-S	1.1	+	+	+	+	−	−	−	−
X-Dp # 1144	C-S	1.1	+	+	−	−	−	−	−	−
X-Dp # 1337	C-S	1.4	+	+	+	+	+	−	−	−
X-Dp # 1186	sc^8	1.6	+	+	−	−	−	−	−	−
X-Dp # 1346	C-S	2.0	+	+	+	−	−	−	−	−
X-Dp # 1328	C-S	2.1	+	+	+	+	−	−	−	+
X-Dp # 1488	C-S	2.5	+	+	+	+	−	−	−	+
X-Dp # 1343	C-S	2.6	+	+	+	+	−	−	−	+
X-Dp # 856	C-S	3.0	+	+	+	−	−	−	−	+
X-Dp # 1498	C-S	3.3	+	+	+	+	−	−	−	+
X-Dp # 3	C-S	4 +	+	+	+	−	−	−	+	+

TEXT-FIGURE 2.—Nature of X duplications. [Reprinted from R. F. Grell (*14*).]

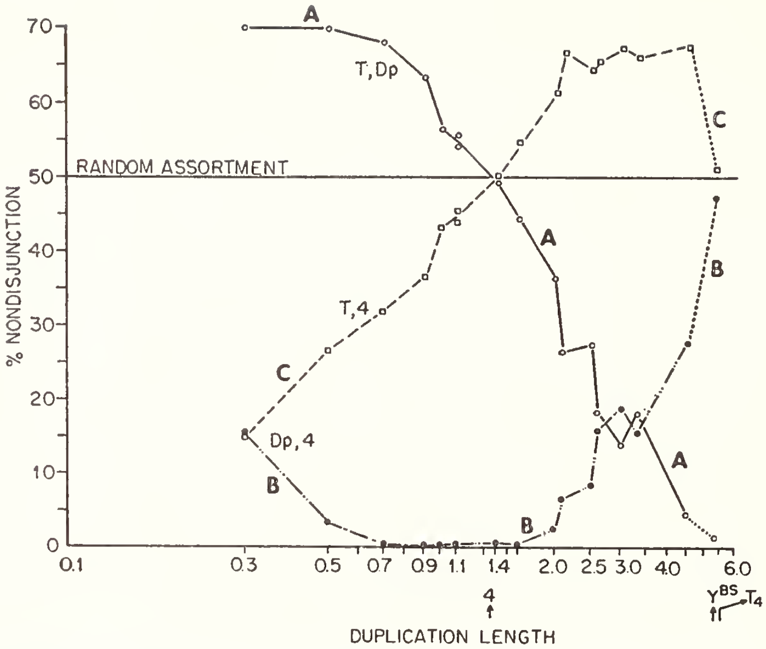
b. Competitive System

The study of the competitive situation may be approached in two ways. The first is to examine the effect of a competitor on the segregation of the two chromosomes that have been studied under noncompetitive conditions. For this it is only necessary to add a third chromosome to the distributive pool. The chromosome that was used, which we call T_4, is 5 to 6 times the length of the four at mitotic metaphase and has been estimated to be a member of the distributive pool, under the conditions of the experiment, about 96 percent of the time. Again, nondisjunction frequencies between the different X duplications and chromosome four were measured. The results are shown in text-figure 3, where the upper curve gives the values when the competitor is present and the lower curve, the values when the competitor is absent. For duplications close to the four in length, *i.e.*, between 0.7 and 1.6, the presence of the competitor has little effect on duplication, four nondisjunction. For duplications smaller than 0.7 and larger than 1.6, the presence of the competitor has a marked effect as evidenced by the sharp rise in duplication, four nondisjunction beyond these points. The effect of the competitor depends, therefore, on the similarity in size of the other two chromosomes.

The second approach permitted a highly critical test of the effect of size on preference in distributive pairing. As indicated, the starting point for these experiments had been the finding that two large chromosomes, with no known homology, segregated regularly from each other, while a small chromosome, with partial homology for one, assorted randomly with both (*18*). This situation is shown in text-figure 4 by the rightmost points on curves A, B, and C. The 2 percent value for curve A represents



TEXT-FIGURE 3.—Comparison of Dp, four nondisjunction in competitive and noncompetitive systems. [Reprinted from R. F. Grell (*14*).]



Text-figure 4.—Relation of nondisjunction frequencies to duplication length. [Reprinted from R. F. Grell (*14*).]

nondisjunction frequency for the two large chromosomes, T_4 and a marked Y; the values of close to 50 percent for curves B and C represent nondisjunction frequencies for the small four and the Y and the four and T_4. In other words, the large Y and large T_4 segregate regularly, while the small four assorts randomly with both.

If pairing preference is a matter of size, it should be possible, by substituting a smaller chromosome for one of the large ones, to alter the preference. Thus, by replacing the Y with X duplications of smaller and smaller size, the point should eventually be reached where the X duplication and the four now segregate regularly and the large T_4 assorts randomly. This point is expected to occur when the size of the X duplication is very close to that of the four. By following curves A and B, we see that this is precisely what happens. As X duplications of smaller and smaller size are substituted for the Y, nondisjunction between the X duplications and T_4, shown in curve A, increases. At the same time, nondisjunction between the X duplication and the four, shown in curve B, decreases. The point is finally reached when the X duplication (Dp 1337—length 1.4) and four associate exclusively, and the T_4 shows 50 percent nondisjunction (random assortment) with both. Actually, this situation resembles the original one, for in both cases the two chromosomes of like size segregate regularly, and the third chromosome, of unlike size, assorts randomly. These results demonstrate that preferences, as well as frequencies, in distributive pairing are size-dependent.

It must be strongly emphasized that pairing behavior at this time is neither a property of the heterochromatic nor euchromatic portions of the chromosome *per se.* The X duplications are predominantly heterochromatic, the T_4 is predominantly euchromatic, while the four has its normal amounts of both. Yet the pairing behavior of the three chromosomes correlates closely with their entire mitotic metaphase length and appears to be independent of the amount of euchromatin and heterochromatin they possess.

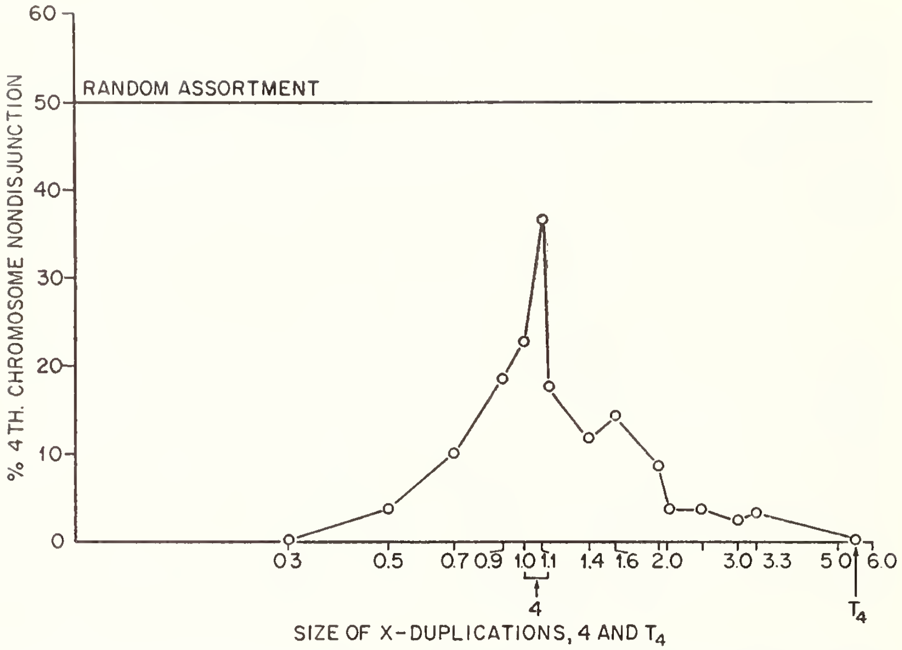
c. Fourth Chromosome Segregation

With the discovery that distributive pairing is size-dependent, it became possible to elucidate the mechanism responsible for the regular segregation of the fourth chromosomes (*26*). According to the distributive-pairing model, the two fours, as noncrossovers, should invariably be members of the distributive pool and available for nonhomologous associations. When other chromosomes are known to be present in the pool, however, there is no evidence for their associations with the fours unless one four has been removed from the pool by involvement in a translocation. As one explanation, it might be postulated that a special mechanism has been evolved to ensure regular fourth chromosome segregation despite the absence of crossing-over and that, as a consequence, the fours, like crossover bivalents, do not enter the pool. The discovery that distributive pairing is size-dependent suggested, as an alternative explanation, that the fours, although pool members, are too small to become involved with other chromosomes of the genome.

To test whether the fours are pool members, it was proposed to introduce the small X duplications into the pool and to determine whether the fours are now susceptible to involvement with a nonhomologue. If so, fourth chromosome nondisjunction should occur.

The results are shown in text-figure 5. Nondisjunction of the fours, which is 0.2 percent with no duplication, steadily increases from a value identical to the control, *i.e.*, 0.2 percent, for the smallest duplication, to a maximum of 36.6 percent for a duplication very close to the four in length (Dp 1144—length 1.1). Since at this point nondisjunction of the duplication and one of the fours is only 26.4 percent, segregation of the nonhomologues is more regular than segregation of the homologues. Then as the duplications become larger than the four, four nondisjunction steadily declines, reaching a value not significantly different from the control (0.3%) with the largest chromosome used, the T_4.

It is evident from these results that the fourth chromosomes are always members of the distributive pool and will associate nonhomologously if a heterologue of the proper size is present. It follows from this that the mechanism responsible for regular fourth chromosome segregation, in the absence of such a heterologue, is distributive pairing. Furthermore, these results demonstrate again that the critical factor in distributive pairing is size, and that, unlike pairing for exchange, homology is apparently inconsequential. Finally, these results suggest that the explana-



TEXT-FIGURE 5.—Fourth chromosome nondisjunction plotted as a function of duplication (and T_4) length. A control value of 0.2 percent was obtained with no duplication. [Modified from R. F. Grell (*26*).]

tion for the phenomenon of preferential segregation of the fourth chromosomes, described by Sturtevant (*27*), may be size differences among the fourth chromosomes.

DISTRIBUTIVE PAIRING IN OTHER ORGANISMS

Although it seems unlikely that the "distributive pairing" mechanism is confined to *Drosophila*, at present there exists no convincing evidence for its occurrence elsewhere. It should be noted that the genetic demonstration of distributive pairing must, in turn, await the genetic demonstration of nonhomologous pairing. Should associations at the distributive phase be restricted to homologues, it would be impossible, genetically, to distinguish between those chromosomes that segregate regularly as the result of distributive pairing and those that segregate regularly as the result of exchange. It is only because distributive pairing in *Drosophila* is not restricted to homologues that it can be recognized. Whether nonrandom assortment of nonhomologues occurs in other organisms is, at present, unknown.

In humans, cytological and genetic information is accumulating at a rapid rate. Here, a number of cases have been reported that lend themselves to the distributive-pairing interpretation. These are situations where rare chromosomal abnormalities of different kinds occur within single families or kinships: for example, a case of an autosomal translocation in one member of a family, and aneuploidy for a sex chromosome in a second; or an autosomal translocation in one member, and aneuploidy for an unrelated autosome in a second; or aneuploidy for an autosome in one member, and aneuploidy for a sex chromosome in another.

This evidence has led J. Valencia and myself (*28*) to propose that the causal event leading to aneuploidy in all of these cases could be nonhomologous association at the distributive phase of meiosis. If this suggestion should turn out to be correct, then the frequency of aneuploidy in the human population might be expected to be related to the frequency of chromosomal rearrangements in human populations.

THE TIME OF EXCHANGE AND DISTRIBUTIVE PAIRING

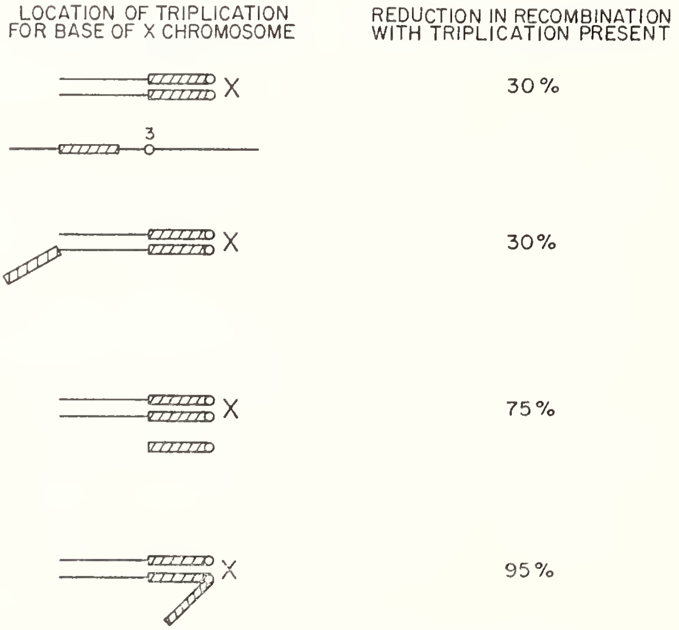
It is appropriate at this point to examine the relationship between the cytologically characterized meiotic stages and the events under discussion. This task would be much simpler if the time of crossing-over were known. Localization of exchange at two different points in the meiotic cycle has led to two alternative interpretations.

The first, or classical interpretation, assumes that cytologically visible pairing between homologues at pachytene represents the pairing that precedes exchange. Since homologous contact presumably occurs along the entire length of the chromosome, the limiting factor is the exchange event, the probability of which for any given interval is considered to be low. With DNA replication occurring well before pachytene, copy-choice as the sole mechanism for exchange is excluded by this model. If this version is correct, exchange pairing would be initiated at the zygotene stage, exchange would occur during pachytene, and distributive pairing any time subsequent to exchange and preceding first anaphase. The speculation that nonexchange univalents might undergo equational division at first anaphase and pair distributively in the secondary oocyte (*29*) cannot be excluded.

The second model, that of Pritchard (*30*), assumes that two types of pairing occur during meiosis. The first is restricted to short discrete regions called "effective pairing" sites; while the probability of establishing such a site is low, the probability of recombination within such a site is high. Recombination is thought probably to occur by a copy-choice mechanism at the time of DNA replication. The second type of pairing, that observed at pachytene, is considered to be a device concerned solely with segregation.

Pritchard's evidence for "effective pairing" sites comes from studies with *Aspergillus* (*31*, *32*) where recombination data of two kinds, 1) localized negative interference and 2) the increase in exchange observed in the presence of a triplicated chromosomal segment, support this hypothesis. When the same kinds of data are examined in *Drosophila*, little if any support for localized negative interference is found. Moreover, early studies of Rhoades (*33*) and Dobzhansky (*34*) have shown that triplicated segments, such as Pritchard used, decrease rather than increase recombination values. Recently, E. H. Grell (*35*) completed a series of experiments in which a triplication (duplication in *Drosophila* terminology) for the base of X existed either free in the genome or attached at different locations (text-fig. 6). In all cases, a marked reduction in exchange in the triplicated segment was observed, but the extent of reduction depended on the proximity of the triplication to its homologous region. If the triplication is attached to the distal tip of X or inserted in chromosome 3, the reduction is only 30 percent; if it is free, the reduction is about 75 percent; but if it is attached to the base of X, very close to its normal location, a 95 percent reduction occurs. Clearly, the availability of an additional site hinders rather than increases recombination.

Interestingly, in *Drosophila*, the triplication itself rarely participates in exchange, whereas in *Aspergillus*, Pritchard reports that the triplicated segment is involved as frequently in exchange as the two normal homologues. In *Aspergillus*, as in *Drosophila*, the intervals proximal to the triplicated segment, in both the normal chromosomes and in the nonhomologous chromosomes carrying the triplicated segment, show a marked reduction in exchange, a result hardly in accord with the "effective pairing" site hypothesis. In line with this, the occurrence of positive interference over long intervals in *Drosophila*, as well as most organisms, becomes very difficult to account for with small discrete pairing sites.


LOCATION OF TRIPLICATION FOR BASE OF X CHROMOSOME
REDUCTION IN RECOMBINATION WITH TRIPLICATION PRESENT
X
30 %
3
X
30%
X
75 %
X
95 %

TEXT-FIGURE 6.—Reduction in crossing-over with location of triplication. [From E. H. Grell (*35*).]

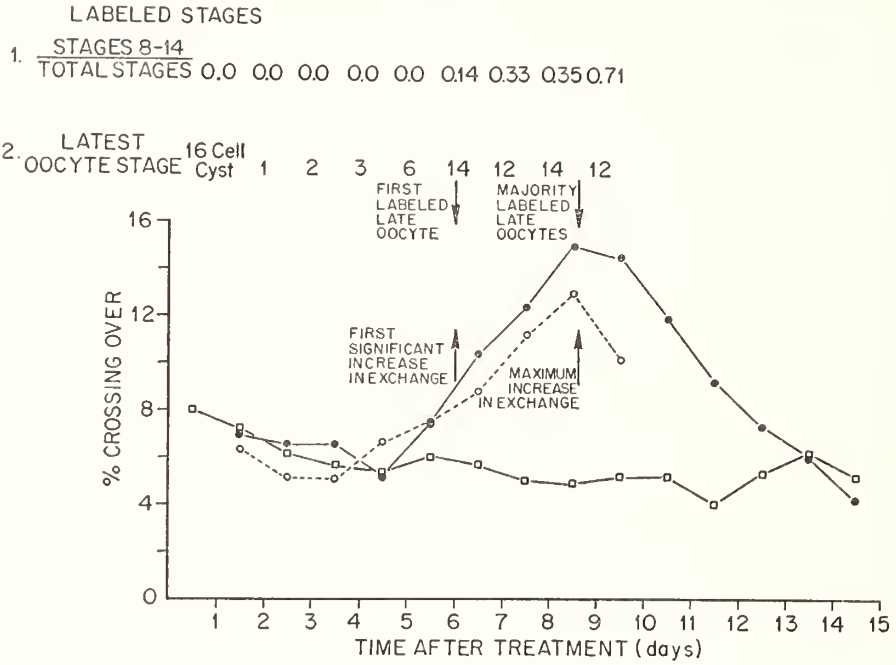
Although Pritchard's model finds little support in *Drosophila*, prepachytene exchange remains an attractive hypothesis. An alternative explanation may be considered (*36*). In addition to the *Diptera*, where homologues are expected to lie in close proximity at the premeiotic telophase because of somatic pairing, there exists a variety of observations in the cytological literature indicating that pairing of homologues in the conventional side-by-side manner is initiated at premeiotic anaphase or telophase (*37*). Chromosome condensation followed by parasynapsis of homologues soon after nuclear fusion has also been described for *Neurospora* by McClintock (*38*) and Singleton (*39*). If this should turn out to be the general rule, then the paired condition necessary for exchange, as well as a paired condition which could account for positive interference, has been established well before meiotic prophase commences.

Recently, in collaboration with Ann Chandley, who has been visiting in our laboratory, I have been attempting to obtain more precise information concerning the time of crossing-over in *Drosophila*. We have used heat treatment of 35° C for a 24-hour period to increase the frequency of crossing-over. We brooded daily thereafter, for 15 days, to determine the time interval between treatment and the deposition of those eggs from which progeny arise with an increased frequency of crossing-over.

In conjunction with the genetic studies, we have injected both heat-treated and untreated females with tritiated thymidine and prepared daily samples of the ovaries to follow the progression in labeling from the early oocyte, where the last DNA replication occurs, to the mature oocyte. In this way it should be possible to determine the time interval between the last DNA synthesis and egg laying.

Our findings are shown in text-figure 7. Those eggs laid approximately 6½ days after treatment produce the first progeny to show a significant increase in exchange. The increase reaches a maximum of about 3 times control value on day 8½, and then falls, reaching the control value again on day 12½. Interestingly, when we study the labeling sequence, we find that label appears in stage 8–14 oocytes for the first time on the 6th day after injection, and the majority of labeled oocytes do not reach stages 8–14 until 8 to 9 days after injection. The correspondence between the times of DNA replication and the alteration in crossing-over by heat indicates, unless the heat effect is a delayed one, that crossing-over is occurring concurrently with or very close to the time of DNA replication. These studies suggest DNA replication occurs in the 16-cell cyst, prior to the stage 1 oocyte.

If exchange pairing and exchange are prepachytene events, associations commenced at zygotene might correspond to distributive pairing. A difficulty with this interpretation has been that the excellent correlation between mitotic metaphase length and segregation behavior suggests that distributive pairing occurs late in prophase or at metaphase when chromosomes are well condensed, rather than at zygotene or pachytene. This difficulty could now be resolved by the recent findings of Moens (*41*), who has shown for the tomato at least, that the traditionally accepted



Text-figure 7.—The correspondence between the times of DNA replication and of temperature-induced increase in crossing-over in the oocyte of *Drosophila melanogaster*. [Reprinted from R. F. Grell and A. C. Chandley (*40*).]

sequence of meiotic prophase stages has probably been misinterpreted. He finds in the anthers, where an age gradient exists, that following interphase, chromosomes first become visible as paired pachytene bivalents; that homologues then begin to separate from each other corresponding to what had previously been thought to be synapsis of chromosomes at zygotene (now renamed schizonema); that separation of homologues continues so that the nucleus becomes filled with a network of threads; and that following this diffuse stage, chromosomes proceed through diplotene, diakinesis, and metaphase.

The events that have been postulated from genetic evidence fit this altered sequence rather well. Pachytene might then represent a continuation of exchange pairing, initiated prior to prophase, schizonema a subsequent stage when chromosomes fall apart unless tied together by a chiasma, and diakinesis or metaphase the time of distributive pairing between condensed chromosomes.

RESUMEN

La meiosis en las hembras de Drosofila ha sido revisada como resultado de recientes hallazgos genéticos. El descubrimiento de que los cromosomas no-homólogos pueden segregar regularmente uno del otro, proveyó de un nuevo medio para explorar las relaciones entre el apareamiento, el crossing-over y la segregación. De estos estudios,

se ha podido establecer que los cromosomas que intervienen en asociaciones no-homólogas representan invariablemente no cross-overs y también que la cantidad de crossing-over entre dos homólogos no es afectada por la participación de uno de ellos en asociación con un no-homólogo. Estas dos conclusiones: al apareamiento de homólogos, que es un prerequisito para que haya intercambio, y el apareamiento de no-homólogos que resulta en una segregación que no es al azar, conducen a la aseveración de que no son acontecimientos competitivos y ocurren en diferentes momentos durante el ciclo meiótico. De este hecho, ha sido formulada la teoría del "apareamiento distributivo" de la meiosis. La característica principal de esta teoría indica dos tipos de apareamiento, uno muy temprano que precede al intercambio, llamado *apareamiento de intercambio* y otro posterior, relacionado con la segregación, llamado *apareamiento distributivo.* El *apareamiento de intercambio* está determinado por la homología; el apareamiento distributivo ha sido demostrado recientemente ser un fenómeno dependiente del tamaño de los cromosomas. La teoría del apareamiento distributivo provee una explicación satisfactoria para un número de problemas hasta ahora sin resolver a la genética de Drosofila incluyendo la segregación regular sin intercambio, el mecanismo de la no-disjunción secundaria, los fenómenos de la distribución no al azar de los no-homólogos y de la segregación preferencial del cuarto cromosoma.

Con respecto al momento de los acontecimientos meióticos, estudios recientes sugieren que el crossing-over puede ocurrir al mismo tiempo o muy cercanamente al momento de la síntesis del ADN. La información disponible sobre Drosofila no apoya la hipótesis de Pritchard del "apareamiento efectivo"; se favorece una alternativa en la cual el apareamiento de intercambio en la forma convencional, lado a lado, se inicia en la anafase o telefase pre-meiótica.

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DISCUSSION

Cordeiro: In crosses between species of the *Drosophila willistoni* cryptic group, we find that from the same population of the *willistoni* and *paulistorum* some strain crosses show most of the cell chromosomes completely paired and others give no pairing at all. This variation in pairing cannot be accounted for by homology, and we suggested that some factor leads to pairing in the early polytene chromosomes when the chromosomes start dividing. But also, it is clear that homology is not necessarily the cause of extensive pairing, since it is not possible to imagine that some strains of *willistoni* are so tremendously close to *paulistorum* and others so completely apart.

Grell: There can be little doubt from studies of the chromosomes of *Drosophila* salivary gland that regular pairing is restricted to regions of homology. The question then is why pairing occasionally fails between homologous regions. This can probably only be answered when we better understand the nature of the pairing forces. We do know that a single gene, as, for instance, the asynaptic gene in maize, can prevent pairing of homologues; but of course here we are dealing with meiotic chromosomes and the pairing forces may be very different from those that cause somatic pairing of the salivary gland chromosomes.

Cordeiro: We cannot use homology as it has been used normally in studying hybrids of these species. Pairing can occur or not depending on some factor, but I do not believe that the *willistoni* group and the other *cryptic* species have such a great homology. It is easy to see that pairing and nonpairing regions appear in similar crosses, which indicates that pairing is not synonymous with homology. That some pairing regions are nonhomologous can be seen in the paired chromosomes because many bands do not go through all the width; they are half-bands only.

Grell: That many bands are half-bands does not necessarily imply lack of homology. If, as suggested recently by some workers (Fujita, S., and Takamoto, K., Nature 200: 494, 1963), bands represent inactive, condensed regions of the chromosome and interbands active regions, then, in the hybrid, the genes in the polytene set from one species may be under different regulatory control from those in the other set and active at a different time from those of the other species. This could result in half-bands even though the paired regions are homologous.

Moses: The possibility that exchange may occur during the paired state at zygotene and pachytene is supported to some extent by the observation of incorporation of tritiated thymidine in pachytene chromosomes in *Triturus* by Wimber and Prensky (Genetics 48: 1731, 1963). This low level incorporation could represent the DNA synthesis (or repair) that should occur during exchange. I would like to suggest a working hypothesis that may serve to tie together some of the cytological and genetic evidence. The first assumption is that chromosomes tend to pair in both somatic and meiotic cells. The second assumption is that a substance is laid down along paired chromosomes, which tends to maintain synapsis. This substance would be the basic protein material which, together with nonhistone protein and DNA, constitutes the three main elements of the axial complex. (Moses and Coleman, The role of chromosomes—development, 23d Growth Soc Symp, Academic Press, 1964, p 11.) Rough pairing, *e.g.*, at the level of chromomeres, would occur independently, while its preservation by the axial material over a period of time could facilitate more precise pairing between homologous DNA molecules in the space down the center of the axial complex. In other words, the synaptenemal complex could provide a system that would permit homologous DNA molecules to find each other and to pair. Genetic exchange could then take place by any of several mechanisms. This would be the region of effective pairing. Nondisjunction might be accounted for by the unusual persistence of the material that makes up the axial complex.

Grell: Do you assume that you may have a persistence of material in the core whether or not exchange occurs, and that the persistence of this material leads to nondisjunction?

Moses: Neither the presence nor the persistence of the synaptinemal material would guarantee exchange, but meiotic crossing over would be unlikely to occur without it. Prolonged persistence of the material provides a mechanism that could account for nondisjunction. We have observed the complex to persist for different lengths of time in different species. Usually, it disappears at diplotene, presumably after having served to facilitate exchange. But, in some organisms it persists in certain regions almost up to what could be described as a prometaphase condition. In these cases, it could tend to keep paired chromosomes together, leading to nondisjunction.

Grell: You have, of course, no genetic evidence for nondisjunction being involved with this? I would find it much easier to believe that the regularly disjoining homologues rather than the nondisjoining homologues are held together by the axial complex, for our genetic evidence clearly shows that it is only exchange chromosomes that remain together and are unavailable for associations with nonhomologues at distributive pairing.

Pavan: You said at the beginning that, with the new data available, the Darlington hypothesis of pairing could be eliminated, and I think that, with this, one could also eliminate the Belling and White hypotheses of crossing-over. I would like you to tell us who demonstrated that the DNA in meiotic divisions is already replicated in the premeiotic interphase. And I would like to ask Dr. Moses for some more details about the hypothesis of this pairing of the DNA molecules and crossing-over. With Darlington, Belling, and White's hypothesis, we could see some mechanical things going on and giving rise to crossing-over; in Dr. Moses' hypothesis I could not see anything of this sort yet. I would like him to say something about this.

Grell: The evidence that DNA replication occurs before pachytene comes from the work of Swift on the mouse (Physiol Zool 23, 1950), Swift and Kleinfeld on the grasshopper (Physiol Zool 26, 1963), Taylor on *Tradescantium* and *Lilium* (Amer Natur, XCI, 1957), and from the recent work of Henderson on the grasshopper (Chromosoma 15, 1964).

Pavan: And no DNA synthesis would occur in prophase?

Grell: As I recall, there is no evidence for DNA replication going on beyond leptotene.

Moses: In two instances (*Tradescantia* and *Trillium*) it has been reported that the major portion of DNA replication occurs during the late interphase, prior to meiotic prophase, but that replication persists into leptotene and zygotene (*Tradescantia*) and into pachytene (*Trillium*; Moses and Taylor, Exp Cell Res 9: 474, 1955; Sparrow *et al.*, Brit J Radiol 25: 182, 1952).

Pavan: And then what would be the sequence there? It starts in late interphase and goes on until when?

Moses: In the cases that I have mentioned, it would continue into the synaptic stages. The aforementioned observations of H^3 thymidine incorporation in pachytene chromosomes by Wimber and Prensky could reflect such DNA synthesis.

Evans: We have three questions up here, and I will ask Dr. Cooper to start.

Cooper: First I would like to say that Dr. Grell's work is simply marvelous and has been an enlivening contribution to its field. Yet I cannot really question her without putting in apposition ideas that I have been working on. I might say, from Dr. Grell's report and my earlier discussions, that some of our findings are simply not understandable at present in terms of one another. In my own work, I became interested very early in what would happen if two X chromosomes could be made as much like a Y chromosome as they are like each other. I was interested in doing so because I wanted to test one of the unstated assumptions of cytogenetics, namely, that synaptic pairing is a reciprocal matter. It might *not* be the case that pairing is reciprocal. It would be in principle, for example, that some one chromosome becomes ready to pair and that the relation then is not reflexive, because it "chooses" a partner and may do so correctly or incorrectly. Now the question is how a notion such as this may be tested. That was the start of the high nondisjunction work. If there is an X, a two-armed Y, and another X, and all three chromosomes are nearly equal partners, then under reflexive pairing (the unstated assumption of meiosis) there should be a maximum of 66 percent secondary nondisjunction. But if there is a temporal order in pairing, so that these chromosomes become randomly "activated" at different times, or if there is simply no simultaneity, then if X1 is first to pair it might choose as its partner X2, the short arm of the Y, or the long arm of Y, and likewise X2 has these choices, were it first to choose partners. But the long arm of the Y might be the first to pair. It is forbidden to choose the short arm of Y, so YL may choose either X1 or X2, the unpaired chromosome making a YS association (or conversely). So 80 percent of the configurations would be XYX trivalents and 20 percent would be XX bivalents and a univalent Y. From this reasoning we would predict that the maximum secondary nondisjunction of the X chromosomes would be approximately 80 percent. And this is precisely what we find to be the highest amount of secondary nondisjunction that we have been able to obtain. So, it may well be that there is this matter of choice of partners, and that pairing is initially not reflexive.

Secondly, were the mechanism of crossing-over the sole means of holding the chromosome together (and Dr. Grell has shown that it is not), it would have to have a very high frequency and precision to keep zygotic viability high. So presumably there is a second mechanism, and we have recently tried to demonstrate that the heterochromatic region of the X possesses pairing sites, which are in discrete position in the heterochromatic region and are not a property of "heterochromatin" *sui generis.* In any case, these conjunctive sites in the heterochromatic regions are responsible for the association of the X and Y chromosomes at spermatogenesis in the male. Dr. Grell, I think, believes that there is no evidence that a second mechanism of this sort exists in the female, but the duplications she has showed us, and many others, were prepared with the notion of testing this question. The pairing sites are not distributed continuously in the heterochromatic region, and there is none within a length of a single fourth chromosome from the centromere. As the sites appear discontinuously along the length, different size duplications ought to find expression in the female by causing segregational patterns to vary as step functions. For example, if there is no pairing site near the spindle attachment, we ought to get essentially random segregation for the smallest duplication. When one of the conjunctive spots (or "collochores") is included in a duplication, then there should be a jump in segregation, and, at some point of increased duplication length, there should be another jump followed by another jump, depending on just how many conjunctive sites are present and their locations. The confirmed existence of these step functions shows that this second mechanism very likely works in the female.

When we made those duplications, Krivshenko and I selected only those possessing a portion of the left end of the X up to, but not including, the prune locus. That means that some 20 or 30 euchromatic bands occur at the end of each heteorchromatic duplication. An interesting matter is that the smallest duplications acted as random fragments. It might be concluded from this that there is just too short a euchromatic length to pair, but we took a different point of view. We noticed that along Bridges' map of the polytene chromosomes, there is always some one or a few short regions that, by chance, have patterns of bands more similar to those of a corresponding small piece than to those of a piece excised from the set at random. It has been shown that interchromosomal effects are nonpredictive because different regions of chromosomes produce quite different interchromosomal effects, and so we have assumed that the initial act of pairing is that which the cytologist sees as an approach of homologues. The first Darlington and Newton papers showed that in triploids all three chromosomes pair. (Later on that was dropped for a strict one-by-one association.) They do pair but they do not synapse, synapsis being one by one. So we wondered whether chromosomes might not come together and match their patterns as best they can. With an inversion, or a small piece, as in these duplications, band patterns might match to some degree many different parts of the chromosomes, and their behavior becomes predictive only when their pattern-pairing choices have been worked out.

Grell: I do not agree that our findings are not understandable in terms of one another. I think your findings are understandable with my model. First, according to your interpretation, rearrangements introduced into an X cause this chromosome to resemble a Y chromosome. The only way you can increase the resemblance between the X and Y is to introduce sequence of DNA from one into the other. Second, whether pairing is reflexive or not, if the noncrossover secondary exceptions arise from preexchange pairing between the X and either arm of the Y, an inevitable consequence of this pairing must be a correlated reduction in total X exchange. My studies show that no such reduction occurs. Next, I think you overlook one very relevant finding, which is that pairing between nonhomologues appears to be completely dependent on size. I see no conceivable way that you could reconcile the size results with your theory of banding patterns. Finally, I would like to ask you about the smallest fragments, without any pairing sites, acting as random fragments, because in my studies they do not behave in this way but segregate very regularly from the fourth chromosome.

Cooper: Our assumption is that it is always segregating from something, and you find that it segregates from chromosome IV. But it is not segregating from its homologue.

Grell: I have not rejected the possibilty of secondary mechanisims for pairing. I am aware that such things as "collochores" exist between the X and Y in the male. I have not found any evidence for this in nonhomologous pairing, but I have not studied homologous pairing in the female from this aspect.

Cooper: "Collochores" do not seem to play a role in nonhomologous pairing.

Evans: I have one point I would like to raise in connection with the experiment in which you gave heat-shock treatment and observed an increase in recombination coinciding with the time of DNA synthesis. I wonder if you are familiar with the experiments by Lawrence on *Tradescantia* and on *Lillium* in which he did not use heat but used X radiation given during the premeiotic period and scored chiasma frequency at diakinesis. Lawrence found that the frequency of chiasma was reduced if the radiation was given during S and increased if the radiation was given during pachytene. This is in contrast to your results. One further point which I am not certain about is the possibility that in your experiment you are, in fact, looking at DNA label in cells that were in the gonial stage at the time of synthesis and not in the preleptotene stage.

Grell: There is little reason for assuming that heat and X rays should have similar effects, particularly since there is no good evidence that the former causes chromosome breakage. It is for this reason that we used heat in our experiments rather than X ray.

We believe that we can distinguish between gonial and oocyte pickup of label. First, oocytes do not undergo another replication so that label picked up in this stage remains undiluted. Oogonia undergo at least one further replication, at which time the pool of H^3-thymidine is expected to be largely depleted so that a minimal 50 percent dilution should occur. Second, labeled oocytes should, in general, precede labeled oogonia along the ovariole. Ann Chandley, who did the cytological work, could follow a sort of frontier of heavily labeled oocytes through the ovariole.

Peacock: Could you elaborate on the details of application of the thymidine and how long it may have been available. I would also like to comment about the tomato work you mentioned. There have been observations in *Lillium* on the cytologically recognizable stages of meiotic prophase, and these seem to be directly correlated with the length of the anther. We would have to assume that proceeding from pachytene to a leptotene stage would have to result in regression in the size of the anther.

Grell: The H^3-thymidine was injected into the abdomen of newly emerged females with a micropipette. The volume injected was not measured, but the injection was stopped at the first visible swelling of the abdomen. The length of time it remained available was also not known. The experiments of Wimber and Prensky with *Triturus* indicate that the pool of H^3-thymidine is largely depleted within 40 minutes.

Cooper: May I just say one thing about chiasmata? One of the peculiarities of female *Drosophila* is that we know next to nothing of the cytology of the female, and the few metaphase I figures in females that have been presented show no chiasmata. They would have to be regarded as possessing "invisible" chiasmata, and this is one of the curious things about fly cytology. I have not seen the paper by Moens to which you refer. Your description suggests what cytologists have long seen in many cases of meiosis and which some have called the "confused stage," at a time of marked growth of the meiocyte. When the confused stage comes on, the chromosomes are at some stage, often beginning diplotene; when they come out of the confused stage they once again continue diplotene changes. So it seems to be a period of relaxation of the chromosomes during pronounced metabolic activity, but it does not seem to alter the sequence of meiotic events as you suggest.

Grell: The possibility that Moens is suggesting is that the "diffuse" stage and leptotene may be one and the same. If leptotene (diffuse stage) follows pachytene rather than preceding it, as suggested by Moen's finding that, in the tomato, nuclei in the pachytene stage are immediately preceded by early pollen mother cells with interphase nuclei, the accepted sequence of meiotic events would be altered.

Schultz: There is a problem in the analysis of any of these genetic data in assigning a sequence in time to a set of data which measure frequencies. One might make the opposite kind of hypothesis to Dr. Grell's and say that initially there is a "distributive pairing" due to the association of kinetochores, for example, as a result of the anaphase polarization. We know very little about the homologies of the kinetochores and cannot say much about the nature of this admitted association at the beginning of the process. Subsequently, one would have what is called exchange pairing. Now the exchange chiasma would influence which elements disjoin from each other. Those that have not undergone exchange pairing would be subject to the influence of the previously determined associations of the kinetochores or adjacent regions. This sequence would, I believe, achieve the same results as the reversed sequence proposed by Dr. Grell.

Grell: There seems to be little conflict in our viewpoint. You have said that exchange affects how chromosomes will subsequently disjoin. Therefore we agree that the associations responsible for the final outcome of disjunction, to which we have given the name "distributive pairing," cannot be decided until *after* exchange. This means, of course, that "distributive pairing" cannot *precede* exchange.

We think we know a little more about the homologies of kinetochores and adjacent regions than you say. We have looked for such homology as a factor in "distributive pairing" and we find it does not exist. What we have found is that the total length of the chromosome is the important factor, as the experiments I have just described have shown.

Schultz: There are two relatively independent events: the association of the elements that determine distribution and the occurrence of exchange, a superposed factor necessary for the final decision. Until both of these decisions are made, you cannot tell what is going to happen. And from the genetic data you cannot tell when which has happened. The final results are the conjunctive result of two separate factors as you have so beautifully demonstrated.

Grell: Perhaps this is a semantic argument. I call the associations responsible for the final decision "distributive pairing." If, as you agree, exchange is necessary for the final decision, then it seems clear this decision cannot be made until exchange has occurred and exchange must *precede* distributive pairing.

You say you cannot assign a sequence in time to data which measure frequencies. These data do more than measure frequencies. They also show that a qualitative difference exists between those chromosomes that can engage in nonhomologous pairing and those that cannot. Those that can are inevitably noncrossovers and those that cannot are inevitably crossovers. And it is the *exchange event* that establishes this qualitative difference. The combination, then, of both the qualitative data, which tell us only noncrossovers can pair with a nonhomologue, and the quantitative data, which tell us nonhomologous pairing does not produce noncrossovers because it does not alter exchange frequency, permits us to assign the sequence. With these two pieces of information, exchange *followed by* distributive pairing becomes a logical necessity.

The Meiotic Mechanics of Ring Chromosomes in Female *Drosophila Melanogaster*[1, 2]

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SUMMARY

The genetic results from 3 kinds of ring chromosomes in *Drosophila melanogaster* were examined: single ring X chromosomes—generated by crossing-over in tandem metacentric and tandem compound ring X chromosomes, heterozygous with a rod X chromosome, and homozygous; tandem compound ring X chromosomes which are 2 X chromosomes arranged in tandem and attached to a single centromere; and reversed compound ring X chromosomes that are 2 complete X chromosomes arranged in reversed sequence and attached to a single centromere. The results indicate: In all ring situations, there is evidence of ring chromosome instability which is detected as ring loss leading either to the production of lethal zygotes or to nullo-X-bearing eggs; at least one important mechanism of ring stability is the process of exchange, because in some situations rings exhibit no loss when exchange occurs, but are lost in the absence of exchange. The evidence for meiotic instability of ring chromosomes, the amount of such instability, and the conditions determining the extent of the instability are considered.—Nat Cancer Inst Monogr 18: 243–273, 1965.

PERHAPS the central fact relevant to a consideration of the mechanics of ring chromosome behavior is that simple three-dimensional considerations suggest that rings should often get into mechanical difficulties during replication and crossing-over. These difficulties, in general, should result in the formation of mutually interlocked ring complexes, which it would seem should in turn result in chromosome breakage and, hence, loss of rings and/or lethality. There are, however, two considerations which might suggest that ring chromosomes would not be subject to any special mechanical difficulties: Primarily, in most cases, and possibly in all circumstances, replication and crossing-over *could* occur in such a way

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[2] Supported by research grant RG-9965 from the National Institutes of Health, Public Health Service.

[3] I wish to express my appreciation to Mrs. Averil Rosenfeld and Mr. Joseph Orem for their invaluable assistance in the collection of the new data reported herein. I also thank Dr. D.L. Lindsley of the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, for many stimulating and helpful conversations about the problems considered.

as to avoid any of these difficulties. But this would represent some special adaptation of the ring to ring structure, and since ring-shaped chromosomes do not form part of the normal genome of any species, it would seem that natural selection could not have adapted ring chromosomes to the requisite special modes of replication and crossing-over. Thus, there is, in fact, no reason to believe, *a priori*, that rings would not get into at least some of the difficulties that one can envision. A second consideration, however, is that even though ring chromosomes would form mutually interlocked complexes, these would have no genetic consequences because the physical structure of chromosomes is such that rings could simply "pass through" each other, unchanged and unharmed, so that the interlocked complexes would be only a transient phase of nuclear division with no ultimate genetic consequences (as is, in fact, suggested by the motion pictures of Bajer). This consideration, too, seems to involve, at least initially, insuperable difficulties because such a structure—one that would permit the passing of one chromosome through another—is hardly consonant with, for example, the observed behavior of anaphase bridges, in which the chromosomes behave like fairly rigid bodies with some elasticity. One sees the chromosomes, for example, in late anaphase bridges, very attenuated and looking like a pair of semielastic ropes stretched between the two poles, a concept that is reinforced by the genetic consequences of anaphase bridges, as elucidated by Sturtevant and Beadle (*1*) and Novitski (*2*, *3*). Such structures would not appear capable of passing through one another.

In summary, it seems reasonable to suppose, as a first approach, that rings could not have become adapted through natural selection to the avoidance of interlocked complexes, and that the interlocked complexes should lead to detectable genetic difficulties. But the most striking feature of ring chromosome behavior is its remarkable stability. Indeed, one conventionally deals with the theoretical aspects of ring chromosome behavior as if the system were just two-dimensional.

In considering the observed stability of rings, we must first distinguish between meiotic and mitotic stability and also between rings in maize compared with those in *Drosophila* (rings have been studied extensively only in these two forms). Generally, it may be said that rings are stable both mitotically and meiotically in *Drosophila* and unstable, in both cases, in maize. However, an exception exists in both—a mitotically stable ring (*4*) in maize, and in *Drosophila*, a mitotically unstable ring [*see*, for example, Hinton (*5*)]. In this discussion, we will confine our attention to the meiotic behavior of (the more general class) mitotically stable rings in females of *Drosophila*.

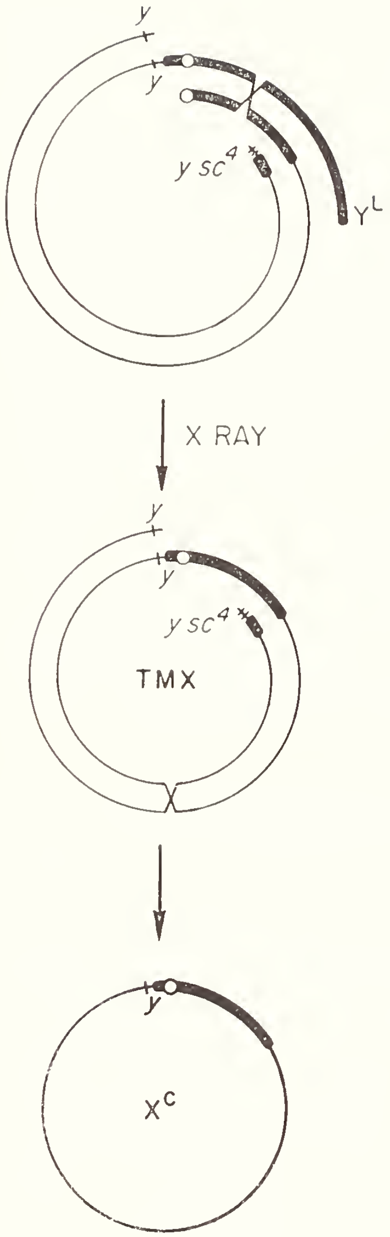
The results from genetic studies on the meiotic behavior of 3 kinds of ring X chromosomes in *Drosophila* have been examined. The 3 types of rings are: 1) single ring X chromosomes; 2) reversed compound ring X chromosomes that are ring chromsomes composed of 2 complete X chromosomes in reversed sequence attached to a single centromere and which, therefore, pair in meiosis in much the same way as attached-X chromosomes; and 3) tandem compound ring X chromosomes that are ring chromosomes composed of 2 complete X chromosomes in tandem sequence attached to a single centromere which, therefore, pair in meiosis in a spiral and consequently generate, upon crossing-over, single ring X chromosomes. The genetic results from such chromosomes suggest 3 important general conclusions: 1) All of the rings are remarkably stable meiotically, given the large number of theoretical difficulties possible; 2) this stability, however, is not as complete as previously believed in that there are circumstances in which any *Drosophila* ring behaves unstably; and 3) the data indicate that one, but not the only, important mechanism for extricating rings from these meiotic difficulties (or, possibly, for the avoidance of them) is the process of crossing-over itself. Consequently, much of the remarkable meiotic stability of *Drosophila* rings may be due to the fact that in most genetic circumstances crossing-over occurs in most tetrads in most meioses.

Obviously, ring chromosomes in *Drosophila* males, also apparently stable, and the mitotic stability of *Drosophila* rings must be due to other stabilizing mechanisms. These matters will not be considered here except to note that meiosis in male *Drosophila* is radically different from that in females, and hence it would be surprising indeed if the same stabilizing mechanisms should exist in the two, while the distinction between meiotic and mitotic chromosome mechanics is self-evident.

SOURCES OF RING CHROMOSOMES

Single Ring X Chromosomes

One can study single ring X chromosomes in *Drosophila* in three circumstances, each of which has special and interesting properties. In the first place, one can study lines carrying "established" single rings, *i.e.*, single rings from a single ring-bearing stock. The first single ring was discovered by Morgan (*6*) in the progeny of an attached-X-bearing female. This ring presumably arose by some kind of exchange between the tip of one arm of the attached-X and the base of the other arm. Established single rings may be studied either heterozygous with a rod X chromosome (ring/rod heterozygotes) or homozygous. In general, however, it might be objected that the very fact that a ring line can be established implies the ring is stable, but it might be imagined that this property is not general. Information on this point can be obtained by examining the progeny of tandem metacentric compound X-bearing females. A tandem metacentric compound X chromosome (TMX) is composed of 2 complete X chromosome elements completely inverted with respect to each other and attached to a single, medially positioned, centromere. The compound thus pairs in a spiral, and a single crossover

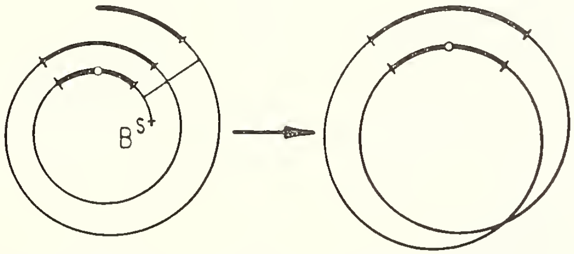


Text-figure 1.—The origin of the single ring X chromosomes used in these studies. The ring results from an exchange in a tandem metacentric compound X chromosome, the origin of which, from the component elements, is likewise shown [after Lindsley and Sandler (7)].

between 2 homologous chromatids attached to the same centromere will generate a single ring chromosome. The origin of single ring chromosomes from tandem metacentrics is shown in text-figure 1. The progeny of TMX chromosomes thus provides a way to study newly generated ring chromosomes prior to the opportunity for any selection.

Tandem Compound Ring X Chromosomes

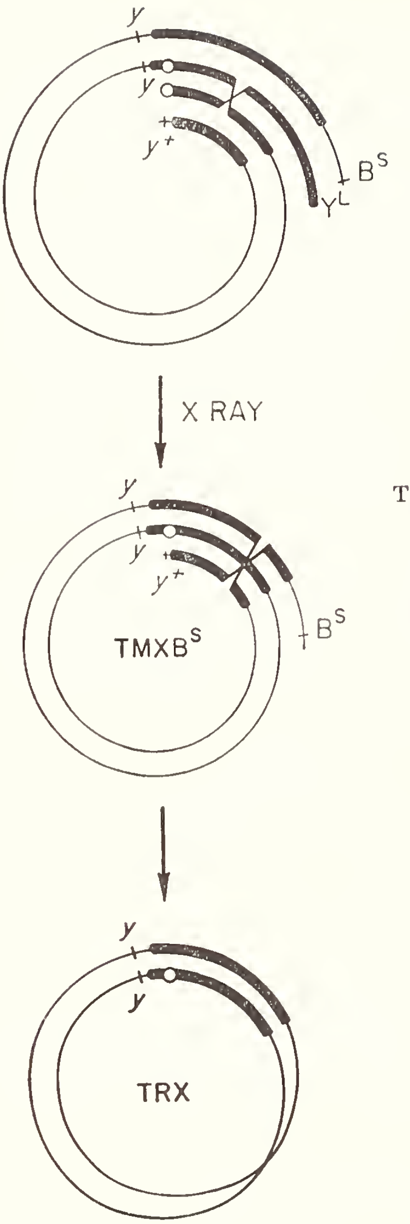
Tandem compound ring X chromosomes (TRX) are ring chromosomes composed of 2 X chromosomes arranged in tandem and attached to a single centromere. They provide the opportunity for studying: 1) the behavior of large ring X chromosomes, 2) newly generated single ring chromosomes, similar to the situation with tandem metacentric compounds, and 3) the process of crossing-over between the elements of a compound in a ring. Tandem rings have been synthesized in two different ways. The first TRX was constructed by Novitski (*8*) from a tandem acrocentric compound X chromosome (TAX=2 X chromosomes attached to each other in tandem and to a single subterminal centromere) with the proximal portion of T(1,4)B^S attached as a second arm of the compound. The B^S duplication contains the proximal heterochromatin of the X chromosome and the euchromatin up to and including the *B* locus and is marked by an extreme allele of *B*. A euchromatic exchange between the B^S duplication and the distal portion of the TAX yields a tandem ring. A schematic representation of this construction is shown in text-figure 2. Another TRX was achieved by Sandler and Lindsley (*10*) by constructing a TMX with a distally attached B^S duplication, and effecting an exchange between the duplication and the distal portion of an arm of the TMX, which yields a TRX as one product. This mode of synthesis is shown in text-figure 3.



Text-figure 2.—The origin of a tandem compound ring X chromosome from a tandem acrocentric compound X chromosome with the proximal portion of T(1,4)B^S appended as a second arm of the tandem acrocentric compound [after Lindsley and Sandler (*9*)].

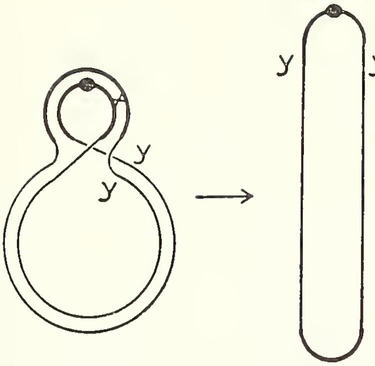
Reversed Compound Ring X Chromosomes

Reversed compound ring X chromosomes (RRX), ring chromosomes composed of 2 complete X chromosome elements attached to a single centromere and arranged in reversed sequence, have been constructed in two different ways. The first reversed ring occurred as a stabilization of a TRX (*8*) effected by a heterochromatic exchange which may be visualized as shown in text-figure 4. A similar stablization of the tandem ring was produced by Sandler and Lindsley. A second, and more direct, construction of the RRX has been achieved by Sandler (*11*), through the construction of an attached-X chromosome, both arms of which con-

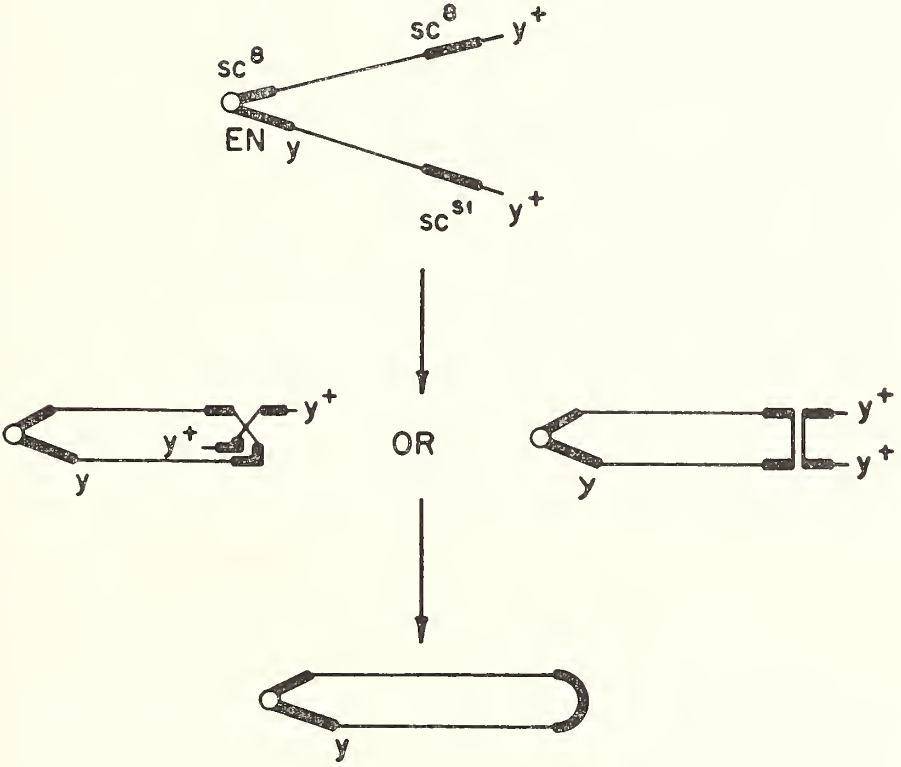


Text-figure 3.—The origin of tandem compound ring X chromosomes from a tandem metacentric compound X chromosome to which the proximal portion of T(1,4)B^S has been appended distally on one of the arms. The origin of the tandem metacentric compound X chromosome, from its component-free X chromosome elements, is also shown [after Sandler and Lindsley (*10*)].

tain distally placed heterochromatin and the normal allele of *y* distal to this. A simultaneous loss of both tips of the attached-X (*i.e.*, a one-step loss of the y^+ phenotype) yields an RRX. This mode of construction is shown in text-figure 5. Reversed rings offer an opportunity, analogous to the TRX, to study ring chromosome behavior in large, double-sized, rings and to study crossing-over between the elements of a ring chromosome, in a situation in which the products of exchange are very different from those of tandem rings.



Text-figure 4.—The origin of reversed compound ring X chromosomes from a heterochromatic exchange in a tandem compound ring X chromosome [from Novitski (*8*)].



Text-figure 5.—Two possible modes of origin of reversed compound ring X chromosomes from an attached-X chromosome homozygous for complete inversions of the X chromosomes and distally placed heterochromatin [from Sandler (*11*)].

THE QUESTION OF SISTER-STRAND CROSSING-OVER

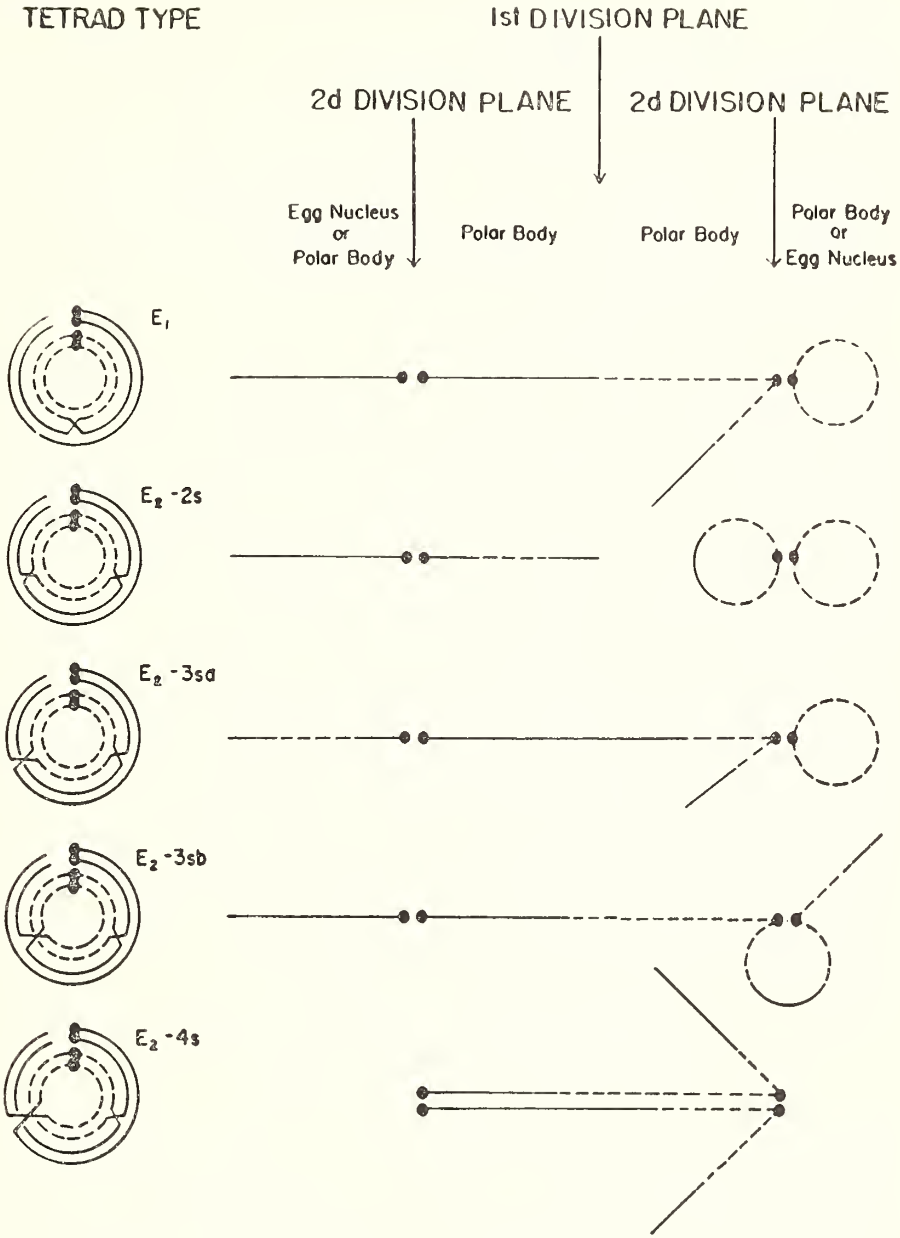
It has been clear since the investigations of Weinstein (*12*) that the relationship between crossing-over, *i.e.*, the result of analyzing single strands of the meiotic tetrad, must be related to exchange (the recombination events in the meiotic tetrad itself) in such a way as to be the algebraic equivalent of supposing that crossing-over occurs in the four-strand stage and that there is no chromatid interference. But Lindegren and Lindegren (*13*) and Schwartz (*14*) have shown that these conditions are satisfied by two different sets of postulates. The first, and conventional set of postulates, supposes that sister strands never cross over, that there is no "strand preference" (any 2 nonsister strands cross over equally frequently) and that there is no chromatid interference (any 2 nonsister strands may cross over at any one level and the strands involved in the exchange at any one level do not influence the strands involved in exchange at any other level). The other set of postulates supposes complete strand preference (in which only the two "newly replicated" nonsister strands can cross over), and, as a corollary, complete chromatid interference, *i.e.*, only two-strand double exchanges occur. However, here one supposes that sister strands cross over with sufficient frequency so that in any region the probability of an effective sister-strand crossover (that is, 1 or an odd number of sisters) is equal to one half.

Although these two sets of postulates give rise to an identical array of expectations for all chromosomes with free ends, they are distinguishable in the case of ring chromosomes. Unfortunately, however, it is found that while the results from ring chromosomes in *Drosophila* [*e.g.*, Morgan (*6*) and Sandler (*11*)] are incompatible with the sister-strand postulates and consistent with the conventional assumptions, ring chromosomes in maize [*e.g.*, McClintock (*15*, *16*) and Schwartz (*14*)] give results more consistent with the sister-strand postulates than with the conventional ones. Although the issue here is unquestionably a basic one, because there is no new information and the arguments suggesting that in *Drosophila* specifically the sister-strand assumptions are untenable, we will, without additional argument in this report, treat the data from *Drosophila* rings using equations based on the conventional assumptions.

SINGLE RING X CHROMOSOMES

Single ring X chromosomes can be analyzed in 3 situations: 1) heterozygous with a rod X chromosome—a tetrad analysis for ring/rod heterozygotes (text-fig. 6); 2) homozygous—the genetically distinct exchanges in homozygous rings (text-fig. 7) and the relative frequencies and consequences of these exchanges (table 1); and 3) newly generated by crossing-over in tandem metacentric compound X chromosomes—a tetrad analysis for TMX's (text-fig. 8 and table 2).

First we will consider the behavior of rings generated by tandem metacentrics. Data from crosses of TMX-bearing females are given in table 3. A detailed discussion of TMX compounds is found in Lindsley and Sandler (*7*). The first striking feature about these data is one observed originally by Novitski and Sandler (*18*), namely, that there is a deficiency of recovered single ring-bearing progeny (in either sex) when



Text-figure 6.—The genetic consequences of exchanges of ranks 1 and 2 in females carrying a single ring X chromosome and a rod X chromosome [from Novitski (*17*)].

the female lacks a homologue for the compound. As can be seen from the TMX tetrad analysis (text-fig. 8 and table 2), the recovery of rings depends both on the exchange frequencies and on *c*, the coefficient of nonrandom disjunction which is the probability of recovering a ring from a ring-TMX dyad (*2*). It might at first be imagined that the presence or absence of a

TABLE 1.—Consequences of exchange of ranks 0, 1, and 2 in a female homozygous for 2 single ring X chromosomes*

Rank and frequency	Crossovers involved	Products after AII separation
E_0	None	n.c.o. X^c: n.c.o. X^c: n.c.o. X^c: n.c.o. X^c
E_1	A, B, C, D, or E	n.c.o. X^c: (AI dicentric ring bridge): n.c.o. X^c
$1/4E_2$	A and B	n.c.o. X^c: d.c.o. X^c: n.c.o. X^c: d.c.o. X^c
$1/4E_2$	A and C	n.c.o. X^c: (AI, AII tricentric ring bridges)
$1/4E_2$	A and D	n.c.o. X^c: (AI, AII tricentric ring bridges)
$1/4E_2$	A and E	(2 AI dicentric ring bridges)

*Crossovers involved are shown in text-figure 7.

homologue influences *c* and therefore the proportion of rings produced. That this is not tenable, however, is shown by the results in table 4, from which it can be seen that although the fraction of recovered rings goes down in the absence of a homologue, there is no concomitant increase in the proportion of recovered TMX-bearing, matroclinous females. Since a change in *c* means a change in the recovery of rings relative to TMX's, a reduction in ring recovery not accompanied by an increased TMX recovery implies that this observed depression cannot be due to changes in *c*.

It seems also true that the depressed ring recovery is not due to a change in exchange values because 1) there would probably be a change in the TMX class, which is not observed, and 2) the one direct measure of exchange—the homozygous TMX class—shows very little change. But it does seem that in the absence of the homologue there is a slight, but consistent, reduction in homozygosis (measured relative to total patroclinous males). Thus it would appear that a fraction of newly generated rings from TMX/0 females are not recovered.

The value of *c* can be computed in TMX experiments from the following equations whose derivation is contained in table 2:

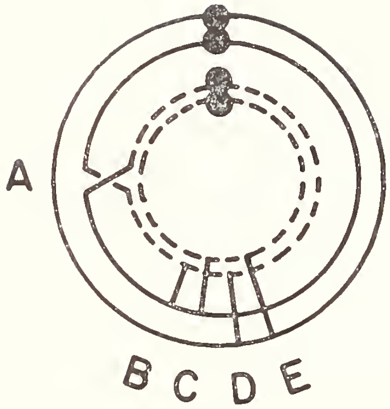
$$\begin{aligned}
&\text{Matroclinous TMX} &&= \tfrac{1}{2}\left[E_0 + \tfrac{1}{2}(1-c)E_1 + \tfrac{1}{8}E_2\right]\\
&\text{Homozygous TMX} &&= \tfrac{1}{2}\left[\tfrac{1}{8}(3-2c)E_2\right]\\
&\text{Rings (in one sex)} &&= \tfrac{1}{2}\left[\tfrac{1}{2}cE_1 + \tfrac{1}{8}(1+2c)E_2\right]\\
&\text{Anaphase II bridges} &&= \tfrac{1}{2}\left[\tfrac{1}{2}E_1 + \tfrac{3}{8}E_2\right]\\
&\text{FR2} &&= \tfrac{1}{2}\left[E_0 + E_1 + E_2\right]
\end{aligned}$$

where E_i is the frequency of tetrads of rank i. It is obvious that if there is reduced recovery of rings in TMX/0 experiments, the computed *c* will have no meaning in those cases. However, *c* varies from line to line even in TMX/FR2 experiments, and, moreover, is negatively correlated with the frequency of exceptional patroclinous males. The exceptional patroclinous male class measures the frequency of eggs that received neither an X nor a Y chromosome, and, therefore, except for a small fraction due to nondisjunction of the compound and FR2, measures potential X-bearing eggs that received no actual product. There is the possibility that in the presence, as well as in the absence, of a homologue newly generated single rings are lost and that 1) such loss leads to the production of nullo-X eggs, and 2) this loss is greater in the absence of a homologue than in its

TABLE 2.—Consequences of exchange and regular disjunction in TMX/FR2 females *

Tetrad types		Products of meiosis and the probability that each will be included in the functional egg nucleus			
Rank and frequency	Crossovers involved	Second meiotic division	First meiotic division	Second meiotic division	
E_0		Matroclinous TMX = 1/4	Matroclinous TMX = 1/4	FR2 = 1/4	FR2 = 1/4
1/2 E_1	A	Ring = 1/2 *c*	Matroclinous TMX = 1/2 (1−*c*)	FR2 = 1/4	FR2 = 1/4
1/2 E_1	B		Single bridge = 1/2	FR2 = 1/4	FR2 = 1/4
1/8 E_2	AC	Ring = 1/2 *c*	Homozygous TMX = 1/2 (1−*c*)	FR2 = 1/4	FR2 = 1/4
1/8 E_2	AD	Ring = 1/4	Ring = 1/4	FR2 = 1/4	FR2 = 1/4
1/8 E_2	AE	Transposed TMX = 1/4	Matroclinous TMX = 1/4	FR2 = 1/4	FR2 = 1/4
1/8 E_2	AF		Single bridge = 1/2	FR2 = 1/4	FR2 = 1/4
1/8 E_2	BC	Homozygous TMX = 1/4	Homozygous TMX = 1/4	FR2 = 1/4	FR2 = 1/4
1/8 E_2	BD	Ring = 1/2 *c*	Homozygous TMX = 1/2 (1−*c*)	FR2 = 1/4	FR2 = 1/4
1/8 E_2	BE		Single bridge = 1/2	FR2 = 1/4	FR2 = 1/4
1/8 E_2	BF		Double bridge = 1/2	FR2 = 1/4	FR2 = 1/4

*Crossovers involved are shown in text-figure 8 [fron Lindsley and Sandler (7)].



TEXT-FIGURE 7.—The genetically distinct exchanges between 2 homologous single ring X chromosomes. The consequences of all relevant combinations of these exchanges are given in table 1.

TABLE 3.—Summary of TMX crosses in which the compound is heterozygous for markers and carries a marked homologue, with data from the same compounds with no homologue*

	Classes of progeny					
	Compound-bearing females		Ring-bearing		Patroclinous males	
	Non-homozygous	Homozygous	Females	Males	Regular	Exceptional
TMX, y/FR2†	1314	234	2135	2407	6230	143
TMXB^{S}9–1/FR2	965	55	975	1040	3382	117
TMXB^{S}9–4/FR2	674	29	994	940	2907	123
RMX, In(1)y^4/+/FR2‡	871	96	1460	0	4241	100
TMX, Hw f/FR2§	567	114	1248	1205	3288	162
TMX, y/0	1254	215	1980	1891	6519	
TMXB^{S}9–1/0	1196	41	748	820	3648	
TMXB^{S}9–4/0	1130	28	1083	881	4175	
RMX, In(1)y^4/+/0‡	1080	100	1240	0	5585	

*From Lindsley and Sandler (*7*).

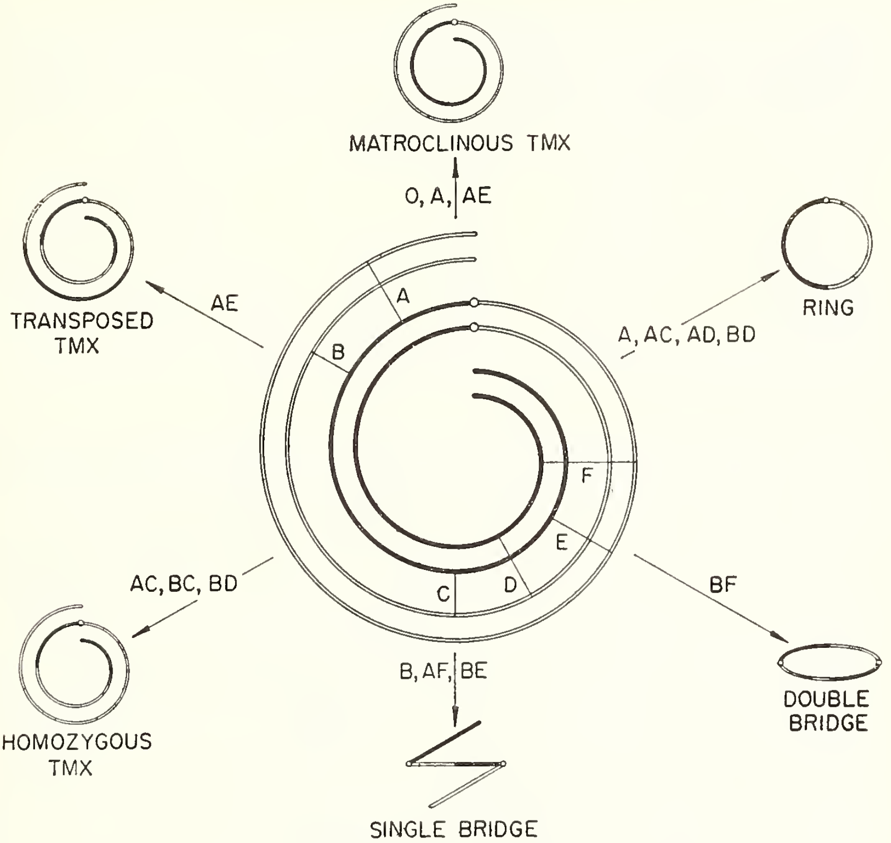
†FR2 is a chromosome equivalent to the long arm of the Y chromosome and is marked by the normal allele of *y* from In(1)sc^8.

‡Data from Novitski and Sandler (*18*).

§Data combined from Novitski (*3*, *17*) as explained in Lindsley and Sandler (*7*); also corrected for unequal production of nullo XY- and XY-bearing sperm (*19*).

TABLE 4.—Effect of the presence of a homologue (FR2) on recovery of ring chromosome derivatives of tandem metacentrics compared with the recovery of tandem metacentrics. [Recovered classes are expressed as the proportion of all patroclinous males (*7*)]

Genotype of female	Total rings	Total tandem metacentrics
TMX, y/0	0. 59	0. 23
TMX, y/FR2	0. 71	0. 24
TMXB^{S}9–1/0	0. 43	0. 34
TMXB^{S}9–1/FR2	0. 58	0. 29
TMXB^{S}9–4/0	0. 47	0. 28
TMXB^{S}9–4/FR2	0. 64	0. 23
RMX, In(1)y^4/+/0	0. 22	0. 21
RMX, In(1)y^4/+/FR2	0. 34	0. 22



Text-figure 8.—The meiotic products of tandem metacentric compound X chromosomes with exchanges of ranks 0, 1, and 2. The tetrad types from which each product may be derived are indicated. The consequences of these exchanges are given in table 2 [from Lindsley and Sandler (7)].

presence. This would explain not only the negative correlation between nullo-X eggs and *c* but also the slight decrease in homozygosis in the absence of a homologue. The effect on homozygosis would come about simply because the patroclinous male class (the standard) is increased in the absence of a homologue by the lost ring class, with, then, the appearance of a decrease in the calculated percent homozygosis.

This hypothesis explains the data shown by the calculations in table 5. With this hypothesis, one can assume just one set of E_i's and one *c* (which, interestingly enough, is approximately unity in every case) for each compound, with only the frequency of ring loss varying with the presence or absence of a homologue. Thus the data suggest that 1) newly generated single rings show instability, 2) the amount of instability is a function of the TMX line generating the rings, 3) the presence of a homologue for the compound markedly decreases the fraction of rings lost, and 4) when a ring is lost, a viable, nullo-X egg results.

TABLE 5.—Estimates of parameters derived from simultaneous fitting of data from TMX/FR2 and TMX/0 to the hypothesis that loss of X^c from presumptive X^c/XY zygotes produces exceptional patroclinous males among progeny of TMX/FR2 females and adds to the patroclinous male class among progeny of TMX/0 females. For a particular TMX, the parameters E_i and c are assumed to be the same for TMX/FR2 and TMX/0 females, but the loss of newly generated rings is assumed to vary*

Compound	E_0	E_1	E_2	c	X^c loss from: TMX/FR2	X^c loss from: TMX/0
TMX,*y*	0. 182	0. 485	0. 333	1. 06	0. 063	0. 181
TMXB^S 9–1	0. 311	0. 571	0. 118	1. 01	0. 107	0. 291
TMXB^S 9–4	0. 291	0. 624	0. 085	1. 09	0. 110	0. 302
RMX, In(1)y^4/+	0. 164	0. 690	0. 146	0. 92	0. 064	0. 324

*From Lindsley and Sandler (7).

In addition to a tetrad analysis of a well-marked TMX, one other kind of information is potentially useful. It is known in *Drosophila* that in the presence of heterozygous autosomal inversions, exchange is increased in the X chromosomes (the "interchromosomal effect"). Accordingly, data were collected from TMX-bearing females, heterozygous for the medially positioned recessive, *v*, and carrying FR2 as a homologue for the compound, with either normal autosomes, or heterozygous for the second chromosome inversions, In(2LR)*Cy*, or heterozygous for the third chromosome inversions, In(3LR)Ubx^{130}, or both (table 6). Although an exhaustive analysis of these data is not appropriate here, it is pertinent to note that while exchange increases with increasing autosomal heterozygosity (as evidenced by an increase in homozygosis), that is, at best, poorly correlated with the exceptional patroclinous male class. This reinforces the conclusion that this class is not, for the most part at least, an exchange class.

TABLE 6.—Results of crosses of females carrying tandem metacentric compound X chromosomes homozygous for *y* and heterozygous for *v*, with FR2 as a homologue for the compound, and carrying autosomally In(2LR)*Cy*, In(3LR)Ubx^{130}, both, or neither, by $Y^sX \cdot Y^L$, *y B*/0 males

Class of progeny	Phenotype of progeny	Autosomal constitution of mother: ++	*Cy* +	+ *Ubx*	*Cy Ubx*
Regular patroclinous ♂♂	*B* ♂♂	2469	1112	1309	1103
Exceptional patroclinous ♂♂	*y B* ♂♂	64	85	119	125
Regular matroclinous ♀♀	*y* ♀♀	570	200	267	247
Exceptional matroclinous ♀♀	+ ♀♀	1	5	4	7
Homozygous ♀♀	*y v* ♀♀	44	20	37	69
X^c/XY ♀♀	*y B* ♀♀	862	281	422	282
Exceptional X^c/XY ♀♀	*B* ♀♀	—	—	1	1
X^c/0 ♂♂	*y* (*v* or +) ♂♂	681	321	348	310
Exceptional X^c ♂♂	+ (*v* or +) ♂♂	2	8	12	4

Once single rings are generated, *e.g.*, by exchange in TMX chromosomes, a ring line can be established and the ring studied in heterozygous ring/rod females. Data from ring/rod heterozygotes have been presented by Morgan (*6*) and Novitski (*17*). New ring data have been collected by

TABLE 7.—Summary of data from females carrying a single ring X chromosome and a normal rod X chromosome

Type of progeny		Source of data		
		Morgan, 1933	Novitski, 1952	This report*
n.c.o.	X^c	3058	4068	2532
	X	4793	4504	3670
d.c.o.	X^c	267	182	128
	X	907	621	432
Patroclinous ♂ ♂		296	313	195

*To make the data comparable with those of the earlier reports, the male classes in the data in table 8, in the *C3G*⁺ column, have been doubled, and the female classes not recorded.

TABLE 8.—Results from crosses of females carrying a single ring X chromosome marked with *y cv v sd* and a wild-type X chromosome, either homozygous for *C3G* or not by (A) In(1)*dl*–49, *y w B*/Y males or (B) $Y^SX \cdot Y^L$, *v f B*/O males

Class of progeny	Phenotype of progeny	Frequency					
		A			B		
		C3G	*C3G*⁺		*C3G*	*C3G*⁺	
X^c/X ♀ ♀	*y B* ♀ ♀ or *v B* ♀ ♀	913	1564		311	1719	
X/X ♀ ♀	*B* ♀ ♀ or *B* ♀ ♀	1269	2082		385	2084	
Exceptional ♀ ♀	+ ♀ ♀ or + ♀ ♀	229	34		61	4	
Patroclinous ♂ ♂	*y w B* ♂ ♂ or *v f B* ♂ ♂	349	195		100	188	
n.c.o. X^c ♂ ♂	*y cv v sd* ♂ ♂	715	1266		211	950	
n.c.o. X ♂ ♂	+ ♂ ♂	1098	1835		454	2143	
d.c.o. X^c ♂ ♂	*y v sd* ♂ ♂	—	3	63	—	2	61
	y sd ♂ ♂	1	13		—	14	
	y ♂ ♂	—	12		—	19	
	y cv sd ♂ ♂	—	18		—	18	
	y cv ♂ ♂	1	11		—	6	
	y cv v ♂ ♂	—	7		—	2	
d.c.o. X ♂ ♂	*cv* ♂ ♂	—	21	216	—	9	265
	cv v ♂ ♂	—	21		—	36	
	cv v sd ♂ ♂	—	49		—	51	
	v ♂ ♂	—	45		—	49	
	v sd ♂ ♂	1	54		—	80	
	sd ♂ ♂	3	26		—	40	

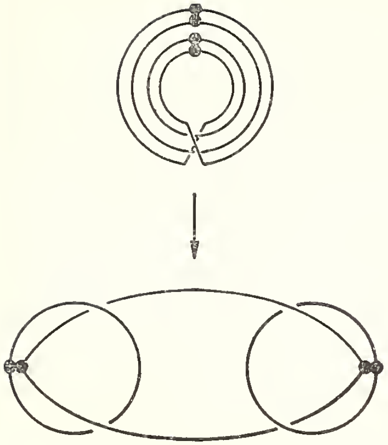
using a ring derived from the TMX discussed previously. A summary of all three sets of data suggesting no special ring difficulties is given in table 7. The ratio of double crossover rings to double crossover rods is not far from the 1:3 expectation [*see* text-fig. 6 and (*6*)], while the patroclinous male class is understood as coming from the asymmetric double anaphase I bridges, the behavior of which has been elucidated (*17*). However, as a consequence of results to be presented later in this report, ring/rod heterozygotes were examined in females homozygous (and, as a control, heterozygous) for the autosomal recessive *C3G*, which almost completely eliminates meiotic exchange. The results from these crosses are given in table 8; the main conclusion is that, when exchange is prevented by *C3G*, rings are apparently recovered less often than the homologous rod X chromosome. The appropriate comparison is as follows. The comparison in which viability complications are minimized is that of

X^c/X versus X/X females. In the *C3G*$^+$ controls there were 1,564 X^c and 2,082 X chromosomes recovered. The male classes tell us that of the 1,564 rings, 74 [(63/1266 + 63) × 1564] were double crossover rings; the others are noncrossovers. Since double crossover rings should be (and, from the results, apparently are) recovered only one third as often as the complementary rod class, the 74 rings are multiplied by 3 to make the ring and rod classes comparable to the *C3G* test where there is virtually no exchange. This means that the X^c/X: X/X ratio should be as 1712:2082, but in fact is only 913:1269; that is, rings are recovered in *C3G* homozygotes only 88 percent as often as in *C3G*$^+$. For experiment B in table 8, this value is 87 percent.

It would be of interest to know, when a ring is not recovered, is a viable nullo-X egg recovered, or is the lost ring an egg lethal. Unfortunately, this is not easy to determine. The 913 recovered rings are 0.88 of the expected number. Thus 125 rings were lost. There were 349 nullo-X eggs, of which at least 229 are complementary to the exceptional female class, which leaves 120 as resulting from lost rings. This is, of course, an excellent agreement. Unfortunately, it is well known that complementary primary exceptional classes are not equal, there generally being an excess of the nullo-X class. Thus, any fraction (and possibly all) of the nullo-X eggs could be primary nondisjunctional, which implies that the lost rings give rise to egg lethals. Although the amount of ring loss here is rather small, it is probably real, since 1) the results are consistent between the two experiments, and 2) *C3G* causes much more striking ring losses of larger ring chromosomes (*see* later).

Finally, single rings may be examined when homozygous. Data from homozygous single ring-bearing females are given in table 9. For this discussion, the most important point is the very large nullo-X egg class, suggesting ring elimination. Text-figure 7 and table 1 show that one eighth of the double exchanges produce detectable double crossover rings of which only 42 were observed. Whatever the fate of the dicentric and tricentric bridges produced by double exchanges, they alone cannot account for the 1,076 nullo-X eggs observed. It must be, then, that rings are eliminated with the resultant production of nullo-X eggs. This might come about either by some ring elimination mechanically similar to that in ring/rod heterozygotes in homozygous *C3G*, or it might be that the anaphase bridge generated by single exchanges can eliminate all 4 ring chromatids. Thus, it is possible to visualize a single exchange in homozygous rings three dimensionally, so as to tie together all of the rings (text-fig. 9).

In summary, then, though the mechanism is always uncertain, it is true that in every circumstance in which single rings can be examined (from TMX's, heterozygous, and homozygous) there is evidence for ring instability—either real, or potential in the absence of exchange—with this instability reflecting itself sometimes, and perhaps always, in the production of nullo-X eggs, but also at times perhaps resulting in egg lethality.



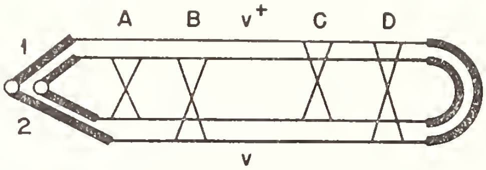
TEXT-FIGURE 9.—A schematic representation of how single ring X chromosome chromatids not involved in a particular exchange, in a tetrad composed of 2 homologous single ring X chromosomes, might be eliminated from the resulting functional meiotic products.

TABLE 9.—Results from crosses of females carrying 2 single ring X chromosomes, 1 carrying the recessive markers *y cv v sd*, and the other marked by *y sn g*, by $Y^SX \cdot Y^L$, *v f B*/0 males

Class of progeny	Phenotype of progeny	Frequency	
X^c/XY ♀ ♀	*B* ♀ ♀	6978	
n.c.o. X^c ♂ ♂	*y sn g* ♂ ♂	2421	4132
	y cv v sd ♂ ♂	1711	
d.c.o. X^c ♂ ♂	*y v sd* ♂ ♂	0	42
	y cv sn g ♂ ♂	0	
	y sn v sd ♂ ♂	3	
	y cv g ♂ ♂	3	
	y sn sd ♂ ♂	5	
	y cv v g ♂ ♂	5	
	y sn g sd ♂ ♂	2	
	y cv v ♂ ♂	1	
	y cv sn v sd ♂ ♂	1	
	v g ♂ ♂	0	
	y cv sn sd ♂ ♂	1	
	y v g ♂ ♂	5	
	y cv sn g sd ♂ ♂	2	
	y v ♂ ♂	2	
	y cv sd ♂ ♂	0	
	y sn v g ♂ ♂	4	
	y cv g sd ♂ ♂	3	
	y sn v ♂ ♂	4	
	y cv v g sd ♂ ♂	1	
	y sn ♂ ♂	0	
Exceptional X^c/X^c ♀ ♀	*y* ♀ ♀	28	
Exceptional $XY/0$ ♂ ♂	*v f B* ♂ ♂	1076	
Equat. X^c/X^c ♀ ♀	*y sn g* ♀ ♀	4	10
	y sn ♀ ♀	1	
	y cv sd ♀ ♀	1	
	y cv v sd ♀ ♀	2	
	y v sd ♀ ♀	2	

REVERSED COMPOUND RING X CHROMOSOMES

The genetically distinct exchanges in the reversed compound ring X chromosome are given in text-figure 10; the relative frequencies and genetic consequences of these exchanges are given in table 10. Data from 3 genetically independently constructed reversed rings are available for analysis. The first is from a stabilization of a TRX, unfortunately without heterozygous markers, effected by Novitski (*8*); the second was a direct construction by Sandler (*11*) from an attached-X and is heterozygous for markers; and the last was from a stabilization of the TRX of Sandler and Lindsley (*10*) and is also heterozygous for markers. Data from these RRX chromosomes are given in table 11, where homozygosis is estimated by following the medially positioned marker, *v*.



TEXT-FIGURE 10.—The essentially different types of exchange theoretically expected in reversed compound ring X chromosomes. Heterozygosity is indicated by the marker *v*. A catalogue of the consequences after exchange is found in table 10 [from Sandler (*11*)].

TABLE 10.—The consequences of exchange of ranks 0, 1, and 2, supposing no sister-strand crossing-over, no strand preference, and no chromatid interference, in the reversed compound ring X chromosome*

Rank and frequency	Crossovers involved	Products after anaphase II separation
E_0	None	Reversed ring + reversed ring (*v*/+) (*v*/+)
1/2 E_1	A or D	Reversed ring + reversed ring (*v*/+) (*v*/+)
1/2 E_1	B or C	AII bridge
1/4 E_2	A and C	AII bridge
1/4 E_2	A and D	Reversed ring + reversed ring (*v*/+) (*v*/+)
1/4 E_2	B and C	Reversed ring + reversed ring (*v*/*v*) (+/+)
1/4 E_2	B and D	AII bridge

*Crossovers involved are shown in text-figure 10 [from Sandler (*11*)].

From these data, the following points can be made: As previously noted (*11*), the presence, in reversed ring-bearing females, of FR2 as a homologue for the compound approximately doubles the frequency of double-exchange tetrads as measured by the rate of homozygosis. There are apparently two different types of RRX's, one of which is exemplified by the Novitski 1954 and Sandler 1957 compounds and shows an over-all sex ratio (females/males) of 0.34 in both cases; the second type, synthesized in the same way as Novitski's original ring (although from an indepen-

TABLE 11.—Results from crosses of females carrying reversed compound ring X chromosomes with or without a homologue (FR2) by $Y^SX{\cdot}Y^L$, *y B*/0 males

Source of data	Genotype of mother	Class of progeny				
		Reg. Pat. ♂♂	Exc. Pat. ♂♂	Reg. Mat. ♀♀	Exc. Mat. ♀♀	Homo. *v* ♀♀
Novitski, 1954	RRX/FR2	3951	391	1443	13	*
Sandler, 1957	RRX/0	814	—	228	—	15
Sandler, 1957	RRX/FR2	12, 034	1159	3956	9	469
This report	RRX/0	2112	—	904	—	19
This report	RRX/FR2	5158	544	2577	2	124

*This compound ring was not heterozygous for any markers.

dently produced TRX), gives an over-all sex ratio of 0.47. That all these chromosomes are reversed rings has been confirmed cytologically, both in larval neuroblast and salivary gland preparations.

The first type of compound ring has been analyzed in two ways (*11*), one of which is no longer valid. When this analysis was first performed, advantage was taken of the speculation (*3*) that anaphase II bridges composed of 2 separating sister centromeres connected by 2 equal-sized chromatin strands would not be included in either of the products of the second meiotic division. Such bridges, therefore, would give rise to nullo-X eggs which are detected, in a marked homologue experiment, as exceptional patroclinous males. Thus in a compound chromosome with heterozygous markers, the frequency of double exchanges could be estimated, both by a female class (homozygous females) and by a male class (exceptional patroclinous males resulting from equal anaphase II bridges resulting from a known fraction of double exchanges), and from this an estimate of the recoverability of rings (at least from double-exchange tetrads) could be obtained. In those experiments, the two estimates of E_2 were equal, which was taken as evidence favoring Novitski's speculation about the fate of equal anaphase II bridges and as evidence that the compound chromosome was completely recoverable—at least from double-exchange tetrads. More recent information (*7*) on TMX compounds makes this reasoning suspect, since the source of the exceptional patroclinous males is now assumed to be lost single rings, as indicated earlier. Nevertheless, one can still argue that the conclusion reached is valid, by noting that the completely independently derived RRX chromosomes of Novitski, 1954, and Sandler, 1957, give an almost identical array of progeny; therefore, it seems safe to assume that the chromosomes suffer no gross viability depression.

If no viability correction need be made, estimates of exchange values can be obtained, by first noting that single exchanges in RRX chromosomes are of two different, equally frequent, types. The first, an exchange between chromatids attached to sister centromeres, yields anaphase II bridges, whereas the second type, exchange between chromatids attached to the same centromere, yields a phenotypically unchanged reversed ring but with altered coupling relations ("transposes"). The coupling relations among RRX-bearing progeny of RRX-bearing mothers—the coupling relations among whose markers were known—were determined, and it was found that the proportion of transposes was no greater than could be accounted for by transposes from double-exchange tetrads (with E_2 estimated from the frequency of homozygosis). It was therefore concluded that the frequency of E_1 was approximately zero. This being so, and since the frequency of double-exchange tetrads is directly estimated from the frequency of homozygosis, the exchange frequencies were known (since $E_0 + E_1 + E_2 = 1$). The estimates are: $E_0 = 0.63$, $E_1 = 0$, and $E_2 = 0.37$. Although this is, of course, a most atypical frequency distribution of tetrads, it is similar to the distribution observed in the structurally similar reversed acrocentric compound X chromosome (*20*); this question has been discussed (*21*, *22*).

The point pertinent to the present discussion is that this analysis is possible without making use of the total number of eggs laid by the compound-bearing females (a number measured by the regular patroclinous male class). If the analysis is correct, and if there were no further complications, then one can derive an expected number for this class. This expectation is not realized. There are many more regular patroclinous males than can be accounted for. This means, biologically, that although the reversed ring suffers from no viability complications owing to its genetic composition, it nevertheless is not completely recoverable. When this analysis was first performed, it was supposed, for the reason just given, that the deficiency of rings could not come from the double-exchange class, and, since there was no single-exchange class, only rings from the no-exchange tetrad class were exhibiting reduced recoverability. Accepting this conclusion, one can estimate the fraction of rings recovered from zero-exchange tetrads. This fraction is 29 percent. Although the initial reason for supposing that this ring loss must come exclusively from the no-exchange tetrad class is no longer valid, again it seems that the conclusion is because it leads to a prediction which is independent of the fate of anaphase bridges (and this is the only product whose fate has been called in question). Since E_1 is approximately zero, then the absence of a homologue for the compound, which results in a reduction of double exchanges by a factor of 2, must be compensated for by a known increase in the E_0 class. Since, on these assumptions, the change in the frequency distribution of tetrads due to the presence, as compared with the absence, of a homologue is known, and since the recovery of each class is also known, one can predict the sex ratio in RRX/0 crosses from the observed homozygosis in those crosses plus the data and inferences from the RRX/FR2 crosses. This prediction is almost exactly satisfied (*11*).

In summary, then, in spite of the uncertainty now attached to the source of the exceptional patroclinous male class, independent arguments suggest that in the first type of RRX chromosome, single exchanges are virtually absent, double exchanges range from 37 percent with a homologue to half that value without—the remaining tetrads being no-exchange, and that among the no-exchange tetrads, rings are recovered only 29 percent as often as expected.

In the newly synthesized reversed ring, the situation is radically different (as indicated immediately by the difference in over-all sex ratio). When one performs a transpose analysis in this chromosome in a manner similar to, but unfortunately less exact than, the analysis performed on the earlier synthesized RRX, it is found that transposes, and hence single-exchange tetrads, are very common. A tetrad analysis in this case follows the same rules as before (from table 10 and text-fig. 10)

RRX-bearing ♀♀ $= 1/2\ E_0 + 1/4\ E_1 + 1/8\ E_2$
Homozygous RRX ♀♀ $= 1/8\ E_2$
Lethal zygotes $= 1/4\ E_1 + 1/8\ E_2$
Regular patroclinous ♂♂ $= 1/2\ (E_0 + E_1 + E_2)$.

The numbers in table 11 are corrected as follows: In the Sandler 1957 compound, there were 469 observed *v* homozygotes, but 1,126 total homozygous females. Thus the 124 *v* females in the RRX/FR2 crosses in table 11 correspond to 298 total homozygotes. Since the regular matroclinous class must be concomitantly reduced, there are:

RRX-bearing ♀♀ = 2403
Homozygous RRX ♀♀ = 298
Regular patroclinous ♂♂ = 5158,

which corresponds to: $E_0 = 0.05$, $E_1 = 0.72$, and $E_2 = 0.23$.

In this case, the frequency distribution of tetrads is reasonable, and, moreover, there cannot be any extensive ring loss because assuming ring loss is to assume a lower E_1, and this is not consistent with the observed high frequency of transposes.

Thus there are two conclusions from these RRX data. It is clear that reversed ring lines can be characterized either by an abnormal distribution of exchange ranks or by a much more usual distribution (although, interestingly, in either case the homologue increases homozygosis to the same extent). 2) But more to the point here is that while reversed ring compounds in which exchange has occurred are evidently recovered as often as expected, reversed ring compounds in the absence of exchange are recovered much less often than expected. The obvious implication from this, as in the case of single rings in ring/rod heterozygotes homozygous for *C3G*, is that the process of exchange itself either rescues ring chromosomes from loss or prevents the difficulties that would ultimately lead to loss from occurring.

It then becomes of interest to inquire into the behavior of the completely recoverable reversed rings in a situation in which exchange is entirely prevented. We first tested this by comparing the recovery of reversed rings and the complementary patroclinous male class when reversed rings are made homozygous for *C3G*. The results of such crosses are given in table 12. It is evident that from females homozygous for

TABLE 12.—Results of crosses of females carrying reversed compound ring X chromosomes homozygous for *y* and a normal Y chromosome, either homozygous for *C3G* or, as a control, heterozygous for *C3G* by $\mathrm{Y^{S}X \cdot Y^{L}}$, *y B*/0 males

Class of progeny	Phenotype of progeny	Frequency	
		C3G	$C3G^{+}$
Patroclinous ♂♂	*y B* ♂♂	473	1069
Matroclinous ♀♀	*y* ♀♀	191	554

TABLE 13.—Results of crosses of females carrying reversed compound ring X chromosomes homozygous for *y* and FR2, either homozygous for *C3G* or, as a control, heterozygous for *C3G* by $\mathrm{Y^{S}X \cdot Y^{L}}$, *y B*/0 males

Class of progeny	Phenotype of progeny	Frequency	
		C3G	$C3G^{+}$
Regular partroclinous ♂♂	*B* ♂♂	129	2038
Regular matroclinous ♀♀	*y* ♀♀	74	982
Exceptional patroclinous ♂♂	*y B* ♂♂	86	148
Exceptional matroclinous ♀♀	+ ♀♀	13	2

C3G, RRX recovery is very significantly reduced. It is obvious, of course, that the $C3G^{+}$ control does not give an expected sex ratio; the expectation in the absence of exchange is a recovery of RRX-bearing females equal to the complementary patroclinous male class.

It is of interest to inquire here, as in the case of single ring loss with *C3G*, whether when a ring is not recovered is the egg lethal or is it a viable nullo-X egg. To test this, crosses were made of RRX/FR2-bearing females, homozygous or not for *C3G*, by $\mathrm{Y^{S}X \cdot Y^{L}}$, *y B*/0 males (table 13). While in this experiment, as in marked homologue experiments in general, the nullo-X class that should have received an X chromosome is distinguishable from the nullo-X class that is complementary to the compound X by the absence of the homologue in the former. A different complication is the increase in nondisjunction (which, of course, also produces a nullo-X, nullo-Y class) due to *C3G* as evidenced by the increase in the exceptional matroclinous female class. Again, unfortunately, as with single rings, a definitive answer relative to the fate of lost rings is not possible.

The data are consistent with the notion that a lost ring results in a recoverable nullo-X, nullo-Y egg. This can be shown in a number of ways. Most simply, under this assumption, 87 RRX's were recovered of which 13 were exceptional. Therefore, to the 129 FR2-bearing eggs recovered (as regular patroclinous males), we add 13 of the exceptional patroclinous males, the remaining 73 being added to the RRX class. This yields 142 eggs complementary to the RRX and 160 (RRX + potential RRX) eggs. This is a good agreement, given the manipulations carried out.

It is, however, possible to show more strikingly that these data will fit the hypothesis that ring loss yields recoverable nullo-X, nullo-Y eggs. We may suppose that in a homozygous *C3G* female, disjunction is regular

x of the time and random $(1-x)$ of the time, and that in either case rings are lost (and nullo-X eggs produced) in a fraction $(1-s)$ of the cases.

Then:

$$B\ \male\male = \tfrac{1}{2}x + \tfrac{1}{4}(1-x) + \tfrac{1}{4}(1-x)(1-s) = \tfrac{1}{4}\,[2-s(1-x)]$$
$$yB\ \male\male = \tfrac{1}{2}x(1-s) + \tfrac{1}{4}(1-x)(1-s) + \tfrac{1}{4}(1-x) = \tfrac{1}{4}\,[2+s(1+x)]$$
$$y\ \ \female\female = \tfrac{1}{2}xs + \tfrac{1}{4}(1-x)s = \tfrac{1}{4}\,[s(1+x)]$$
$$+\ \ \female\female = \tfrac{1}{4}(1-x)s = \tfrac{1}{4}\,[s(1-x)].$$

Since these equations depend only $s(1-x)$ and $s(1+x)$, these quantities may be estimated by the method of maximum likelihood and then estimates of x and s obtained. These are: $s = 0.55$ and $x = 0.67$. These estimates yield an array of expectations that agree with the data by the χ^2 test. That is, the *C3G*, RRX/FR2 data are consistent with the assumption that disjunction is regular 67 percent of the time, that the ring is lost a little more than half the time, and that this ring loss results in the production of recoverable nullo-X, nullo-Y eggs.

Although the data are consistent with this hypothesis its validity is by no means certain. It is well known that the complementary classes of nondisjunction are not recovered equally frequently; there is always an excess of the nullo versus the diplo class. The amount of this discrepancy varies sufficiently so that no prediction in any specific case is possible. In this particular case, this means that any fraction (or even all) of the *yB* males could be simply nondisjunctional, and thus, an ever-increasing fraction (up to all) of the ring losses would have to be assumed to result in egg lethals. Thus, with RRX ring loss, as with single ring loss, the consequences of such loss must remain equivocal.

Certainly, the most unifying assumption at this time would be that reversed ring loss always leads to lethality since this must be true for the RRX with a high frequency of E_0 (table 11). In the Sandler 1957 RRX line, there were 12,034 presumptive RRX-bearing eggs. Of these, 63 percent had no exchange and of these 61 percent were lost (*see* previous calculations). Thus there were 4,700 rings lost, but only 1,159 exceptional patroclinous males recovered.

In summary, then, reversed rings 1) are completely recoverable when exchange takes place, 2) show a markedly reduced recovery in the zero-exchange class—the lost rings being egg lethals, and 3) show a markedly reduced recovery when homozygous for *C3G* (and therefore $E_0 \approx 1$), where it can be assumed that ring loss results either in egg lethality or in the production of nullo-X eggs.

Although all preceding arguments involved the source of the exceptional patroclinous male class, it now seems clear that these males do not come exclusively from equal double bridges and their source remains obscure. By varying exchange in RRX's by the use of autosomal inversions, we hoped to determine at least whether the exceptional patroclinous male class increased with exchange. Use was made, as in the case of TMX's, of In(2LR) *Cy*, and In(3LR) *Ubx* ^{130} (table 14).

TABLE 14.—Results of crosses of females carrying reversed compound ring X chromosomes homozygous for *y* and FR2 as a homologue, and carrying autosomally In(2LR)*Cy*, In(3LR)*Ubx130*, both or neither, by $Y^S X \cdot Y^L$, *yB*/0 males

Class of progeny	Autosomal constitution of mother			
	+ +	*Cy* +	+ *Ubx*	*Cy Ubx*
Regular patroclinous ♂♂	7544	5871	5326	3200
Exceptional patroclinous ♂♂	970	816	969	588
Regular matroclinous ♀♀	4324	3147	1961	762
Exceptional matroclinous ♀♀	12	83	33	21

The exceptional patroclinous male class does go up with autosomal inversion heterozygosity, but not very strikingly. Unfortunately, as there is no independent measure of exchange, a clear conclusion about whether these males are from an exchange class is not possible.

TANDEM COMPOUND RING X CHROMOSOMES

The genetically distinct exchanges in the tandem compound ring X chromosome (TRX) are shown in text-figure 11; the genetic consequences, and their relative frequencies are given in table 15. There exist data from 3 independent constructions of TRX chromosomes: the TRX of Novitski, 1954, and 2 TRX's that differ in having come from 2 different TMX lines, TMB^{s}9–1 and TMB^{s}9–4 *(10)*. Data from crosses of females carrying these 3 chromosomes are summarized in table 16. The behavior of TRX chromosomes in general has been discussed *(10)*.

Among the inferences possible from an examination of these data, the most important, for a consideration of ring stability, is that TRX lines differ in the success with which the tandem ring is transmitted. This

TABLE 15.—The consequences of exchange of ranks 0, 1, and 2 in the tandem compound ring X chromosome, supposing no sister-strand crossing-over, strand preference, or chromatid interference *(8)*. The coefficients c_1 and c_2 are the probabilities of recovering a single ring from a single ring-double ring dyad and single ring-triple ring dyad, respectively*

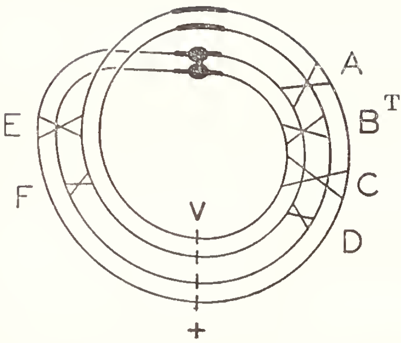
Rank and frequency	Crossovers involved	Products after AII separation
E_0	None	het. dbl. ring (1/2) + het. dbl. ring (1/2)
1/2 E_1	A, B or E	sgl. ring (c_1) + het. dbl. ring (1–c_1)
1/2 E_1	C, D or F	3:1 bridge
1/8 E_2	A + E	het. dbl. ring (1/2) + het. dbl. ring (1/2)
1/8 E_2	D + F	homo. dbl. ring (1/2) + homo. dbl. ring (1/2)
1/8 E_2	A + F	sgl. ring (c_2) + triple ring (1–c_2)
1/8 E_2	C + E	sgl. ring (c_2) + triple ring (1–c_2)
1/8 E_2	B + E	sgl. ring (1/2) + sgl. ring (1/2)
1/8 E_2	B + F	2:1 bridge
1/8 E_2	C + F	1:1 bridge
1/8 E_2	D + E	2:1 bridge

*Crossovers involved are shown in text-figure 11.

Table 16.—Results of crosses of females carrying homozygous *y* tandem compound ring X chromosomes, with and without FR2 as a homologue, by $Y^SX \cdot Y^L/0$ males

Source of data	Source of TRX	Genotype of mother	Classes of progeny						
			Reg. pat. ♂♂	Exc. pat. ♂♂	Reg. mat. ♀♀	Exc. mat. ♀♀	Reg. X^c/X ♀♀	Reg. $X^c/0$ ♂♂	$X^c/FR2$ Excs.
Novitski, 1954	TAX	TRX/0	9790*	—	735	—	2195*	2095	—
Novitski, 1954	TAX	TRX/FR2	3012*	302*	245	3	924*	913	—
Sandler and Lindsley, 1965	TMB^S9-1	TRX/0	203	—	1	—	54	40	—
Sandler and Lindsley, 1965	TMB^S9-4	TRX/0	15, 912	—	2611	—	3404	1675	—
Sandler and Lindsley, 1965	TMB^S9-4	TRX/FR2	7836	1127	1430	5	3096	2667	5

*Numbers corrected for "meiotic loss," according to Sandler and Braver (*19*).



TEXT-FIGURE 11.—The essentially different types of exchange theoretically expected in the tandem compound ring configuration. Heterozygosity is indicated by the marker *v*. A catalogue of the consequences after exchange is found in table 15 [after Novitski *(8)*].

follows from the fact that the proportion of single ring X chromosomes (generated by crossing-over in the compound) is the same for all 3 TRX lines (the proportion of single rings is taken relative to the patroclinous male class). This strongly suggests that the exchange frequencies are the same for the different lines. However, the recovery of the compound ring itself, *i.e.*, the regular matroclinous female class, varies from almost zero in the TRX from TMB^s 9–1 through an intermediate value for the TRX of Novitski 1954 to a maximum value for the TRX from TMB^s 9–4. This almost certainly does not merely reflect differences in the viability of the rings, because the TRX showing intermediate recoverability (the line of Novitski 1954) is the same chromosome that generated a reversed ring line having normal viability. (The argument for the normal viability of this RRX has been given in the preceding section.)

The TRX from TMB^s 9–4 was heterozygous for a number of recessive markers which permitted a tetrad analysis (*10*). The exchange values for this line, and, by extrapolation, for TRX's generally, are very similar to the other tandem compounds, *i.e.*, tandem metacentric compound X chromosomes (*7*) and tandem acrocentric compound X chromosomes (*23*). Given the exchange values, one could show that the TRX was completely recoverable. Thus, the conclusion of most consequence here is that TRX recovery among compounds having the same exchange distribution can vary from almost zero to approximately 100 percent and that this is a property of the line itself, since it characterizes a particular construction of the compound.

As with single rings and reversed compound rings, we can ask whether the compound, completely recoverable when most tetrads have at least one exchange, is equally recoverable in the absence of exchange (that is, homozygous *C3G*). The results of TRX crosses with and, as a control, without *C3G* are given in table 17. It is evident from the data that, as with the other rings in the presence of *C3G*, ring recovery is markedly decreased.

Finally, in the case of tandem rings, as in all of the other compound analyses, the source of the exceptional patroclinous male class is difficult to determine. Thus, it was again hoped that use might be made of the effect of autosomal inversions on increasing exchange frequency to at

TABLE 17.—Results of crosses of females carrying tandem compound ring X chromosomes homozygous for *y* and a normal Y chromosome, either homozygous for *C3G* or, as a control, heterozygous for *C3G* by $Y^SX\cdot Y^L$, *y B*/0 males

Class of progeny	Phenotype of progeny	Frequency	
		C3G	$C3G^+$
Patroclinous ♂♂	*y B* ♂♂	1062	2797
Matroclinous ♀♀	*y* ♀♀	350	690
X^c/XY ♀♀	*y B* ♀♀	0	892
X^c/0 ♂♂	*y* ♂♂	0	811

TABLE 18.—Results of crosses of females carrying tandem compound ring X chromosomes homozygous for *y* and heterozygous for *v* and FR2, as a homologue for the compound, and carrying autosomally In(2LR)*Cy*, In(3LR)Ubx^{130}, both or neither, by $Y^SX\cdot Y^L$, *y B*/0 males

Class of progeny	Phenotype of progeny	Autosomal constitution of mother			
		+ +	*Cy* +	+ *Ubx*	*Cy Ubx*
Regular patroclinous ♂♂	*B* ♂♂	2686	3021	2367	1493
Exceptional patroclinous ♂♂	*y B* ♂♂	330	522	453	423
Regular matroclinous ♀♀	*y* ♀♀	552	319	271	57
Exceptional matroclinous ♀♀	+ ♀♀	1	18	4	4
Homozygous ♀♀	*y v* ♀♀	32	49	22	15
X^c/XY ♀♀	*y B* ♀♀	1085	1156	922	612
Exceptional X^c/*XY* ♀♀	*B* ♀♀	—	19	7	3
X^c/0 ♂♂	*y* (*v* or +) ♂♂	830	931	734	502
Exceptional X^c ♂♂	+ (*v* or +) ♂♂	2	21	15	5

least inquire whether the exceptional patroclinous male class increased with increasing exchange values. Data in table 18 show that, as with similar data for tandem metacentrics and reversed rings already given, the situation is equivocal. The amount of homozygosis and the frequency of double exchanges, seem unaffected by the autosomal inversions, and hence the rise (though again not strikingly so) of exceptional patroclinous males with autosomal heterozygosity is difficult to interpret.

In summary then, data from tandem rings suggest two kinds of ring instability. The first is characterized by a variation in the success with which TRX-bearing females transmit the tandem ring to their progeny—a property of the TRX line—and the second is characterized by a reduced recovery of an otherwise completely recoverable ring when exchange is prevented through the use of *C3G*. Since the source of the exceptional patroclinous males in these experiments is difficult to determine, one cannot state whether, when a ring is lost, this gives rise to a viable nullo-X egg or to an egg lethal.

CONCLUDING STATEMENTS

The purpose of the foregoing discussion was to present the evidence and arguments for just a few general conclusions. The first of these is that in every ring chromosome situation (in the meiotic divisions of female

Drosophila melanogaster) there is evidence of ring chromosome instability—either in fact or potentially in the absence of exchange. Thus, single chromosomes newly generated by exchange in tandem metacentric compound X chromosomes will, in a fraction of cases varying with the particular compound line and according to whether the compound-bearing female carries a homologue for the compound, be eliminated from the egg nucleus with the resultant production of nullo-X eggs. Single rings in ring/rod heterozygous females appear to be quite stable, but potential instability exists, as can be demonstrated by preventing exchange through the use of *C3G*, whereupon a reduced recovery of rings relative to rods is observed. Females homozygous for single ring X chromosomes produce a fraction of nullo-X eggs larger than can be accounted for by anaphase bridges involving more than 2 centromeres; thus here too, rings experience special difficulties owing to their ring structure. Reversed compound ring X chromosomes likewise exhibit instability in the absence of exchange; the evidence is of two kinds. There are those reversed ring lines that exhibit an abnormal tetrad distribution characterized by a high frequency of zero and double-exchange tetrads (with few, if any, single exchanges). In these cases, it appears that while all the rings from the multiple-exchange tetrad class are recoverable, this is true for only a small fraction of the zero-exchange tetrad class—the remaining rings being lethal. Other reversed ring lines are characterized by more usual tetrad distributions, and have very few zero-exchange tetrads and no detectable ring loss. But if exchange is prevented with *C3G*, then the fraction of rings recovered is greatly reduced. Finally, tandem compound ring X chromosomes have two different forms of instability. First, the over-all recoverability of the ring varies from tandem ring line to tandem ring line. Secondly, even though, in the absence of *C3G* and a substantial zero-exchange tetrad class, the ring can be shown to have normal viability and recoverability, in the absence of exchange, *i.e.*, in homozygous *C3G* females, only a fraction of the expected rings are recovered.

The second general conclusion from these studies is that the process of exchange itself is an important mechanism for ring stability. It is, evidently, not the only mechanism because single rings, either homozygous or newly generated by tandem metacentric compounds, have instability along with exchange, and, in tandem rings, compound recovery varies from line to line, while the exchange values remain constant. This, coupled with 1) the mitotic stability of rings, and 2) the stability of single rings in males indicate that, even in the absence of exchange, rings are more stable than would be expected on theoretical grounds. However, the evidence presented here indicates that exchange either rescues some rings which would have been lost or else prevents the event giving rise to ring loss from happening.

A third conclusion is that ring loss probably can result from a variety of causes because the amount of loss varies with so many conditions, *e.g.*, the line, the presence or absence of a homologue, and the level of exchange.

Finally, though the data from at least that type of ring loss occurring only in *C3G* females are consistent either with the assumption that nullo-X eggs are produced or that egg lethals result, the loss of reversed rings in no-exchange tetrads in the absence of *C3G* results in egg lethals, while ring loss of newly generated single rings from tandem metacentrics results in nullo-X eggs. Thus, there are at least two mechanisms of ring loss, or possibly, single rings are eliminated from the egg nuclei when they form mutually interlocked complexes, while interlocked compound rings kill the egg. It can, of course, easily be imagined that this happens merely as a consequence of the size difference.

Undoubtedly, these conclusions are very general, but they do, perhaps, indicate the appropriate framework for further considerations about the meiotic mechanics of ring chromosomes in *Drosophila* in comparison with other forms.

RESUMEN

En este trabajo se estudian los resultados de experimentos genéticos con tres clases de cromosomas en forma de anillo en *Drosofila melanogaster.* Estos cromosomas ofrecen la siguiente constitución morfológica: 1) Un solo cromosoma en anillo de varios origenes. 2) Dos cromosomas X en forma de anillo compuesto. Los mismos están dispuestos en tandem y unidos a un centromero simple. 3) Dos cromosomas X completos en forma de anillos compuestos. Estos se presentan invertidos en relación a sus secuencias y están unidos a un solo centromero.

Los resultados obtenidos indican que: 1) En todos los casos, en que esté presente un cromosoma en anillo hay evidencias de que el mismo es inestable. Esto es detectado por pérdida del cromosoma en anillo que a su vez lleva a la producción de cigotas letales o a huevos sin cromosomas X. 2) Un importante mecanismo de estabilidad del cromosoma en anillo es el proceso de intercambio, dado que en algunas situaciones los cromosomas en anillo no muestran pérdidas cuando se produce el intercambio, pero se pierden en la ausencia del mismo.

Se consideran también las evidencias sobre la inestabilidad meiótica de los cromosomas en anillo, la medida de dicha inestabilidad y las condiciones que determinan a la misma.

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DISCUSSION

Lindsley: Have you tested the nondisjunction in C3G females which do not have a ring, to see whether, in fact, there is an equal recovery of reciprocal, exceptional types?

Sandler: No, I have not done that yet, but I certainly shall.

Lindsley: If they were equally recovered, you would have an estimate of ring loss in the X^c;C3G crosses.

Sandler: Yes, if they are equally recovered, then I think one can decide whether ring loss gives rise to dead eggs or nullo-X eggs.

Cooper: Have you studied the stability of the ring Y chromosomes where you have comparatively very small rings? Have you studied these in the meiosis of the female?

Sandler: No, I have not.

Cooper: It might be one way to cancel out what is and what is not an inherent property of a ring chromosome.

Sandler: Yes, I think the ring Y should be studied, and also some of these things should be looked at somatically but I have not done any such experiments yet.

R. F. Grell: Have you thought of using a heterozygous X inversion system instead of C3G? Certainly C3G has effects other than eliminating crossing-over in the X chromosome, such as effects on segregation.

Sandler: Right. The only reason I am bothered by using an inversion is that I am talking here about the frequency of no-exchange tetrads and I do not know how I would distinguish between the lack of observed crossing-over produced by the inversion and no-exchange tetrads. You see I recover 80 percent of the expected single rings, and thus there is only a very small fraction of ring loss to deal with. This would be an excellent thing to do in the larger rings where the amount of loss is much higher, and then I do not know how to get an inversion into one of these very easily. The idea, however, is certainly very reasonable.

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Chromosome Function at the Supragenic Level[1, 2]

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SUMMARY

The genes on a chromosome do not function independently of one another. Spatial relations among genes play an important role in gene action. Evidence from three general types of observation favors this assertion: *Coordinate function:* Genes functionally related may occur close together on the chromosome in a cluster known as an operon that is controlled by a single mechanism. Genes on the same chromosome may be coordinately controlled by the response of the entire chromosome to mechanisms that cause it to be euchromatic or heterochromatic or to be present or absent in different cells. Because they are coordinately controlled, genes on these chromosomes may be functionally related. *Position effect:* A gene in its normal position may function at different levels depending on the genetic constitution of another region on the same chromosome; this region may be contiguous to or some distance from the gene in question. Genes are also known to function abnormally when they are transferred into abnormal chromosomal environments. *Chromosomal continuity:* Certain chromosomal, if not genic, activities are known to depend on the linear chromosomal continuity between specific chromosome regions, and interrupting this continuity by rearrangement or recombination interferes with normal function. These classes of phenomena are interrelated. Exalemps of each type are cited in the text.—Nat Cancer Inst Monogr 18: 275–290, 1965.

THE CHROMOSOME is more than a linear array of independent genetic factors. It is a nuclear organelle differentiated along its length into regions of heavy and light stainability, the heterochromatin and the euchromatin, and having features such as ends, the centromere, the nucleolus organizer, and constrictions. To what extent are the genes on a particular chromosome interrelated in ways more meaningful than that they occur in the same package for transmission to subsequent generations of cell and organism? In this paper, I shall examine the proposition that the chromosome is not only a unit of transmission but is also a unit

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[2] Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

of function, and the actions of its component genes are complexly interrelated.

COORDINATE GENE FUNCTION

Coordinate function of groups of genes is becoming widely known in microorganisms, in which loci involved in the successive steps of particular biosyntheses seem to be controlled as units known as operons. The cistrons in an operon are contiguous, and a polycistronic messenger RNA has been postulated as the unit of function involved (*1*). Examples of coordinate function at a much grosser level of organization that must be explained on entirely different bases are also known. For instance, the genes on the mammalian X chromosome probably function in a coordinate manner. In mammalian cells of various X-chromosome constitutions, all but one of the X's seem to be heteropyknotic and inferentially nonfunctional. Lyon (*2*) has postulated that early in embryogenesis one X in each cell is fixed as the functional X chromosome and the other X (in females or X's in multiple-X karyotypes) becomes heteropyknotic. This is believed to give rise to as many different types of cell clones in the developing organism as there are X chromosomes in the karyotype. Genetic evidence favoring this interpretation derives from the observation that female mice heterozygous for sex-linked mutants are variegated for the phenotype that is characteristic of the mutant in males and homozygous females. In heterozygous females, different sex-linked genes tend to express themselves in the same tissue patches when they are on the same X chromosome but in different patches when they are on different X's (*3*). Finally, normal alleles of autosomal genes, when translocated to the X, variegate for their recessive phenotype in females but not in males (*4*).

More extreme examples of this type of behavior are found in the coccids [*see* Brown and Nur (*5*) for review and literature citations]. One would be that, in the mealy bug after the first several cleavage divisions, half of the chromosome complement of males becomes heterochromatic and remains so but the entire complement remains euchromatic in females. The heterochromatic set is paternally derived because induced chromosome breaks appear only in the euchromatic set after maternal irradiation and only in the heterochromatic set after paternal irradiation. The heterochromatic set is genetically inactive as judged by the following criteria. 1) The survival of daughters of irradiated fathers is quite radiosensitive, whereas survival of sons is almost completely radioresistant. 2) Mutants that behave as recessives in females express themselves in males when maternally, but not when paternally, inherited. The heterochromatic set is not completely without function, however, because males that survive extremely high doses of radiation to the father have more nearly normal amounts of the heterochromatic material than those that die. The sons surviving such high doses are frequently sterile. If the donor of the heterochromatic complement belongs to a different, but closely related, species from the female, the resulting zygotes fail to develop. There are few if any clues about the control of heterochromatization. Since the chromosomes of the coccids are holokinetic, they may be fragmented into many smaller kinetic pieces. Then all fragments of maternally derived chromosomes remain euchromatic and those of paternally derived chromosomes become heterochromatic. Thus the chromosome's capacity to respond to whatever controlling mechanism may exist seems to be uniformly distributed along its length. The chromosomes in artifically produced androgenetic haploids undergo a transitory heterochromatization and then revert to the euchromatic state. The euchromatic set in diploids seems to play a role in maintaining the heterochromatic complement. This role is not an interaction of homologs since the odd chromosome in a monosomic embryo behaves in accordance with its parental origin.

Elimination or diminution of chromosomes during cleavage is another way in which clones of different functional karyotypes may be formed. Two classical examples of this type of behavior are the limited chromosomes of *Sciara* (*6*) and the chromosomes of *Ascaris* (*7*). Limited chromosomes are large, heterochromatic elements found in the germ line of *Sciara coprophila*. They are eliminated from some nuclei in early cleavage, and these nuclei develop into the soma; nuclei in which they are retained give rise to the germinal tissues. In *Ascaris* first cleavage produces two daughter cells having different chromosome behavior. In one, the two large chromosomes that are characteristic of the germ line karyotype break up into a number of fragments, some of which are kinetic and constitute the karyotype of the descendants of the cell while others are akinetic and are lost. The two chromosomes remain intact in the other cell, which at the next division produces one daughter that undergoes diminution and one that does not. This unilateral inheritance of an intact chromosome complement continues for several divisions, after which the one cell with two large chromosomes begins to undergo equal mitosis to produce a clone of such cells that will become germinal tissue; the remaining cells produce the soma.

In the last two cases, a particular subset of the chromosome complement is eliminated from certain cells during development but is maintained in others. Since this chromatin is maintained in the species, it must perform some indispensable function in tissues in which it is retained and yet be clearly dispensable to cell lines from which it is eliminated. It seems reasonable to assume that the constellations of genes on such chromatin control functions peculiar to tissues in which they are retained; for example, one could imagine that the limited chromosomes in *Sciara* are important for normal gametogenesis.

POSITION EFFECT

A second class of phenomena indicating that the genes on a chromosome do not function independently of one another is position effect. The classical example is that of Bar in *Drosophila melanogaster* worked out by Sturtevant (*8*). Bar (*B*) is a tandem repeat of bands 16A1–6 of the salivary gland X chromosome (*9, 10*). It produces an anterior-posterior narrowing of the eye, and the phenotype apparently results from band 16A1 of the right segment being next to 16A6 of the left segment instead of its normal neighbor 15F8. Exchange between the left duplicated section of one chromosome and the right segment of its homolog in homozygous Bar females generates a triplication (known as double Bar, *BB*) and nonduplicated chromatid. Sturtevant noted that the eyes of *B*/*B* females were considerably less narrowed (68 facets per eye) than those of *BB*/+females (45 facets per eye). Yet each genotype carries 4 doses of the 16A1–6 region and two 16A6–1 associations. The only difference is that the new associations are on homologous chromosomes (the *trans* configuration) in *B*/*B* and on the same chromosome (the *cis* configuration) in *BB*/+. Thus the position of the two new associations with respect to each other has a marked influence on the expression of the genes. Since about 1950 such differences in phenotype between the *cis* and *trans* heterozygotes for mutations affecting the same gene have been the stock in trade of the microbial geneticists and have led to the concept of the cistron. But in Bar, this is an effect that extends over 6 salivary chromosome bands, and perhaps the ancient terms of coupling and repulsion are preferable to *cis* and *trans*. The Bar case and the others to be mentioned differ further from the situation within a cistron in that the mutants in coupling rather than in repulsion cause the greater departure from normal phenotype. It has been possible to explain the intracistronic *cis-trans* effects in terms of the production of two singly abnormal gene products in *trans* heterozygotes and of doubly abnormal and normal gene products in *cis* heterozygotes. Such explanations, however, do not apply readily to Bar and similar cases; rather, it seems more likely that events at the level of chromosome organization are involved.

Hannah-Alava (*11*) has described a case in which mutants that are more than 10 map units and about 400 salivary gland chromosome bands apart differ in strength of expression depending on whether they are in coupling or repulsion. She studied two groups of closely linked mutants. The first included *Scx* (Extra sex comb) and *Pc* (Polycomb), both of which are at about 47 on the third chromosome and promote the formation of sex combs (modified bristles found on the first tarsal joint of the prothoracic legs of males) on the mesothoracic and metathoracic legs of males. The second included members of the bithorax group of mutants *bx* (bithorax), *bxd* (bithoraxoid), and *Ubx* (Ultrabithorax) at 3–58.8, which stimulate mesothoracic development of metathoracic structures. She scored the number of extra sex-comb teeth in males of various genotypes. Her results, summarized in table 1, show that mutants at the bithorax locus act as enhancers of both *Pc* and *Scx* and this enhancement is more extreme when the mutants are in coupling than in repulsion.

TABLE 1.—Mean number of extra sex-comb teeth in heterozygous males with the mutant genes in coupling (numerator) compared with those with the mutant genes in repulsion (denominator)*

Genotype with respect to *Pc* and *Scx*	Genotype with respect to bithorax alleles				
	+	$\frac{bx}{+}$	$\frac{bxd}{+}$	$\frac{bx\ bxd}{+\ +}$	$\frac{Ubx}{+}$
$\frac{Pc}{+}$†	2.4	$\frac{8.2}{4.1}$	$\frac{4.4}{2.7}$	$\frac{9.5}{3.0}$	$\frac{6.0}{5.0}$
$\frac{Scx}{+}$	7.1	$\frac{11.6}{10.6}$	$\frac{10.5}{6.9}$	$\frac{15.3}{11.6}$	$\frac{15.1}{18.3}$

*Hannah-Alava (*11*).

†Bristles counted only in males with at least one extra sex-comb tooth.

Gibson and Thoday (*12*) reported a similar case. They studied two loci, at about 27 and 47 on the second chromosome, that affect bristle number. At each locus there are two known alleles, one (+) that causes greater bristle production than the other (−). Their results indicate that, whereas +−/−+ is a viable phenotype, ++/−− dies: +− and −+ are apparently normal chromosomes, ++ has never been recovered in a viable fly, and −− is homozygous lethal. The phenotypes used in this case are not so precise as those of Hannah-Alava, and ways may be imagined in which the conclusions could be spurious. It seems quite probable, however, that the authors have data that would rule out such alternative explanations. Taken at face value, there are two genes for increased bristle number 20 units apart that in repulsion have no adverse effect but in coupling are dominant lethal.

About 10 years ago I encountered a similar case involving structural heterozygotes for the X chromosome. I observed that females heterozygous for an X chromosome carrying the short arm of the Y chromosome appended distally (*i.e.*, $Y^{S}X\cdot$ = FR-1) and an X chromosome appended terminally to the short arm of the Y chromosome (*i.e.*, $XY^{S}\cdot Y^{L}$) rarely produced $Y^{S}XY^{S}\cdot Y^{L}$ recombinants and when such recombinants were recovered they were seldom transmitted to the succeeding generation. Data from sample crosses presented in table 2 show that crossing-over is occurring because there is ample recovery of the reciprocal recombinant type (*i.e.*, $X\cdot$). Furthermore, the data are not compatible with nonrandom inclusion of certain meiotic products in the egg nucleus. If directed segregation at the first division were occurring, there should be unequal recovery of reciprocal non- and double as well as of single recombinants. Reciprocal classes of non- and double recombinants are recovered with equal frequency. In nonrandom disjunction at the second division, as described by Novitski (*13*), homologous centromeres are recovered with equal frequency whereas homologous terminal regions that are heteromorphic are recovered nonrandomly. Both the centromere carrying $Y^S \cdot Y^L$ and the terminus carrying Y^S are recovered in considerably less than half the progeny (table 2). The conclusion is that the $Y^SXY^S \cdot Y^L$ recombinants are produced and, furthermore, they get into the functional egg nucleus with the expected frequency. Their absence from the progeny is attributed to the inviability of the recombinant-carrying individuals. This inviability is observed irrespective of whether the recombinant-X-bearing ovum is fertilized by an X- or Y-bearing sperm; that is, the recombinant behaves as a dominant lethal. When a $Y^SXY^S \cdot Y^L$ is recovered in combination with an appropriately marked normal X chromosome, the female in question may be crossed and the recovery of the $Y^SXY^S \cdot Y^L$ chromosome among her progeny recorded. The results of such crosses are shown in the last line of table 2; recovery of $Y^SXY^S \cdot Y^L$ is still very low, but in this case this chromosome appears among the non- and the double crossover, and reciprocal single recombinant types are recovered with equal frequency. In summary, it may be said that $Y^SX \cdot / XY^S \cdot Y^L$ is a viable genotype whereas $Y^SXY^S \cdot Y^L / X \cdot$, which has the same elements as $Y^SX \cdot / XY^S \cdot Y^L$ but with the Y chromosome arms in coupling rather than repulsion, has extremely low viability. It is not known whether the lethality is the result of defective chromosomal physiology or chromosomal malfunction during early cleavage mitoses. An examination of inviable zygotes from $Y^SX/XY^S \cdot Y^L$ females reveals that they occur with about the frequency expected but there is no sharp time of death; rather, death is distributed over all the stages of embryonic life (M. L. Pardue). The $Y^SXY^S \cdot Y^L$-bearing flies that do survive are completely normal in phenotype and fertility.

Another aspect of position effect seems to depend on the interaction of genes on homologous chromosomes. Lewis (*14*) has shown that the mutant phenotype of bx^{34e}/Ubx (for example) is enhanced if either third chromosome is involved in an aberration with one breakpoint between the bithorax locus and the centromere. He believes that such rearrangements interfere with somatic pairing and consequently with a functional interaction between homologous loci. He has termed this phenomenon the transvection effect, and has suggested that perhaps homologous loci cooperate in the transcription process, possibly by a copy choice mechanism. Another gene whose activity seems to depend on its ability to pair with its homolog is *SD* (Segregation Distorter 2-55) (*15*). When pairing in the primary spermatocyte of *SD*/+ males is normal the *SD*-bearing second chromosome is included in functional sperm nuclei to the virtual exclusion of its homolog, but when pairing is suppressed by heterozygous inversions, *SD* and + are recovered with equal frequency.

A second class of position effect is variegated or V-type position effect. Muller (*16*) first observed this among the progeny of X-irradiated males in his original experiments on the mutagenic effects of irradiation. Variegation may result when genes, or groups of genes, ordinarily situated in a euchromatic environment are transferred to a heterochromatic environ-

TABLE 2.—X-chromosome constitution of progeny recovered from females whose X chromosomes are heteromorphic both proximally and distally for Y-chromosome material*†

Genotype of mother	Progeny									
	♀ ♀				♂ ♂					
	non + dbl		sgl		non		sgl		dbl	
	+−	−+	++	−−	+−	−+	++	−−	+−	−+
$Y^sX\cdot/XY^s.Y^L$ 110–8	1524	1666	28	1937	1221	1283	8	1770	183	152
$Y^sX\cdot/XY^s.Y^L$ 115–9	1792	1973	162	2148	1335	1657	98	1949	208	189
	++	−−	+−	−+	++	−−	+−	−+	++	−−
$X\cdot/Y^sXY^s.Y^L$ 115–9	85	790	458	452	42	689	381	360	4	71

*Non = nonrecombinant; sgl = single recombinant; dbl = double recombinant.

†+− = $Y^SX\cdot$; −+ = $XY^S.Y^L$; ++ = $Y^SXY^S.Y^L$; −− = X.

ment, and, to a lesser extent, when the converse transfer takes place. The gene (or genes) involved functions at different levels in different cell clones in tissues where expression is ordinarily identical for all cells. Evidence indicates that heterochromatic regions do not synthesize RNA (*17*), and Schultz (*18*) postulated that variegation resulted from heterochromatization of the adjacent euchromatic genes. I understand that Dr. Schultz has new observations on this point; perhaps he would care to mention them during the discussion.

Position effect provides many examples of a gene's ability to function depending on its position with respect to other genes in the complement.

CHROMOSOME CONTINUITY

There are observations indicating that the linear integrity of the chromosome is important to proper function. An example of such behavior is neocentromere formation in maize. Rhoades (*19*) has reviewed this subject. In the presence of abnormal chromosome 10 (a tenth chromosome recovered from numerous strains of Indian corn from Latin America and the southwestern United States and having a long heterochromatic segment on the end of the long arm), accessory spindle fibers are formed during meiosis at all heterochromatic knobs in the complement and the knobs precede the centromeres to the poles. Rhoades demonstrated that linear continuity between a knob and its centromere is a prerequisite to neocentric activity of knobs by showing that knob-bearing acentric chromosomes formed by exchange in heterozygotes for a paracentric inversion do not form neocentromeres. He postulated that a substance required for formation of spindle fibers flows from the centromeres along the chromosomes to the knobs.

A final example in which chromosome organization is apparently important to cellular function is fertility in male *Drosophila*. Schultz (*20*) noted that males carrying reciprocal translocations between the X chromosome and the autosomes are frequently sterile, whereas those that carry insertional translocations are usually fertile. My own interest in this problem was aroused when it became apparent that, among sex-linked-recessive position-effect lethals (*i.e.*, lethals that die as X0 males and survive as XY males), those that were associated with inversions and insertions tended to be fertile and those associated with reciprocal translocations between the X chromosome and chromosome two or three were sterile (*21*) (table 3). Furthermore, as shown in table 4, among translocations between the X and the Y chromosome, a large fraction of those that were sterile as T(X;Y)/Y males could be shown to be T(X;Y;A)'s where A represents chromosome two or three, whereas those that were T(X;Y)/Y fertile did not show autosomal involvement (*22*). Not only is there a striking correlation between X; autosome translocation and male sterility, but the phenotype of the sterility associated with such translocations is always the same. The sperm heads fail to elongate and frequently become dissociated from the sperm tails. Heads of the most mature spermatozoa are highly condensed, heavily staining spheres or ellipsoids; they are frequently dispersed within the testis rather than being associated with bundles of sperm tails in groups of 64. All X; autosome translocations that have been examined in the mouse are also male sterile with the exception of one that is suspected of being an insertional translocation (*4*).

TABLE 3.—Classification of variegated-type position effect sex-linked recessive lethals with respect to male fertility and X;autosome translocation*

	T(X;A) present	No T(X;A) present
X/Y ♂♂ fertile	3	13
X/Y ♂♂ sterile	13	1

*From Lindsley, Edington, and Von Halle (*21*).

TABLE 4.—Classification of viable T(X;Y)'s with respect to fertility of T(X;Y)/Y males and autosomal involvement in the translocation*

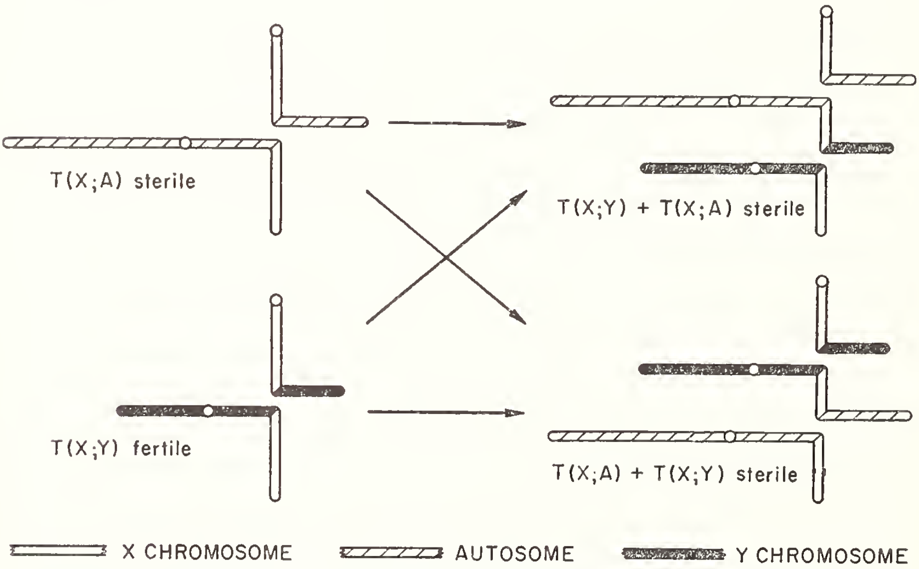
	Polytene cytology of translocation	
	T(X;Y;A)	T(X;Y)
T(X;Y)/Y ♂♂ fertile	0	43
T(X;Y)/Y ♂♂ sterile	13	7

*From Nicoletti and Lindsley (*22*).

The continuing investigation of the relation between X;autosome translocations and male sterility has been a joint one involving Dr. Mary Warters of Centenary College, Shreveport, Louisiana, Mrs. Guthrie T. Pratt, Miss Claire Witten, and Miss Mary C. Stewart, all currently or formerly with the Biology Division of the Oak Ridge National Laboratory, and Mrs. Cloé Musatti, formerly of the Department of General Biology of the University of São Paulo; all these people have contributed to our knowledge of the problem.

It was originally observed (*21*) that the sterility of translocation-bearing males could not easily be attributed to variegated-type position effect or to chromosome-breakage-associated mutation, since both types of events should accompany inversions and insertional translocations as well as reciprocal translocations, whereas male sterility is associated almost exclusively with the latter type of rearrangement. The single phenotype of sterility is a further argument against mutation at many different loci as the cause of male sterility. As a further test of whether recessive changes at the X chromosome breakpoint are responsible for the sterility, a series of 1 lethal and 9 sterile reciprocal T(X;A)'s with X chromosome breaks in lettered subdivisions 2D through 3C were combined with B^sw^+Y, which carries a section of the X chromosome from *kz* (salivary location 2E1-F6) through *dm* (salivary location 3C9-D2). In no case did the presence of the duplication covering the breakpoint of the translocation confer fertility, although the lethal translocation was rendered viable.

Lindsley, Edington, and Von Halle (*21*) advanced the hypothesis that continuity between factors at the base and tip of the X chromosome is required for male fertility and the continuity is interrupted by a reciprocal translocation but not by an inversion or an insertional translocation in which the X is either the donor or recipient of the inserted piece. The fertility of reciprocal translocations between the X and the Y as well as between X and 4 led to the rather contrived amendment to the hypothesis that the centromeric regions of X, Y, and 4 all bear the critical proximal factor. The hypothesis suggested that interposition of a T(X;Y) between the breakpoint of a T(X;A) and the tip of the X would re-establish the critical continuity between distal and proximal factors and should lead to fertility. Among the T(X;Y;A)'s recovered by Nicoletti (*23*), 10 were three-break translocations, 7 of these were of the type Y^DX^P; A^DY^P; X^DA^P, and 3 had two heterochromatic breaks and could not be scored. Furthermore, there were 5 cases in which there was a two-break T(X;A) in addition to a T(X;Y); in 3 the X break of the T(X;Y) was distal to that of the T(X;A), and in 2 it was proximal. In all cases, translocation-bearing males were sterile. Said in another way, a T(X;Y) is male fertile and yet the X is broken into two segments; a reciprocal translocation of either of these segments with an autosome will produce male sterility (*see* text-fig. 1). To cling to the original hypothesis would require the supposition that the X and Y (presumably both arms) chromosomes have comparable material distally as well as proximally and that each half of a T(X;Y) becomes an element whose continuity is important to male fertility.



TEXT-FIGURE 1.—The fertility of combinations of translocations between the X and an autosome and between the X and the Y chromosome.

To further examine the relation between X;autosome translocations and male fertility, Dr. Mary Warters selected a series of X;autosome translocations from the daughters of irradiated males by virtue of the production of apparent linkage between normally unlinked genetic characters (table 5). The table shows that, of those translocations surviving as males, about three quarters are male sterile.

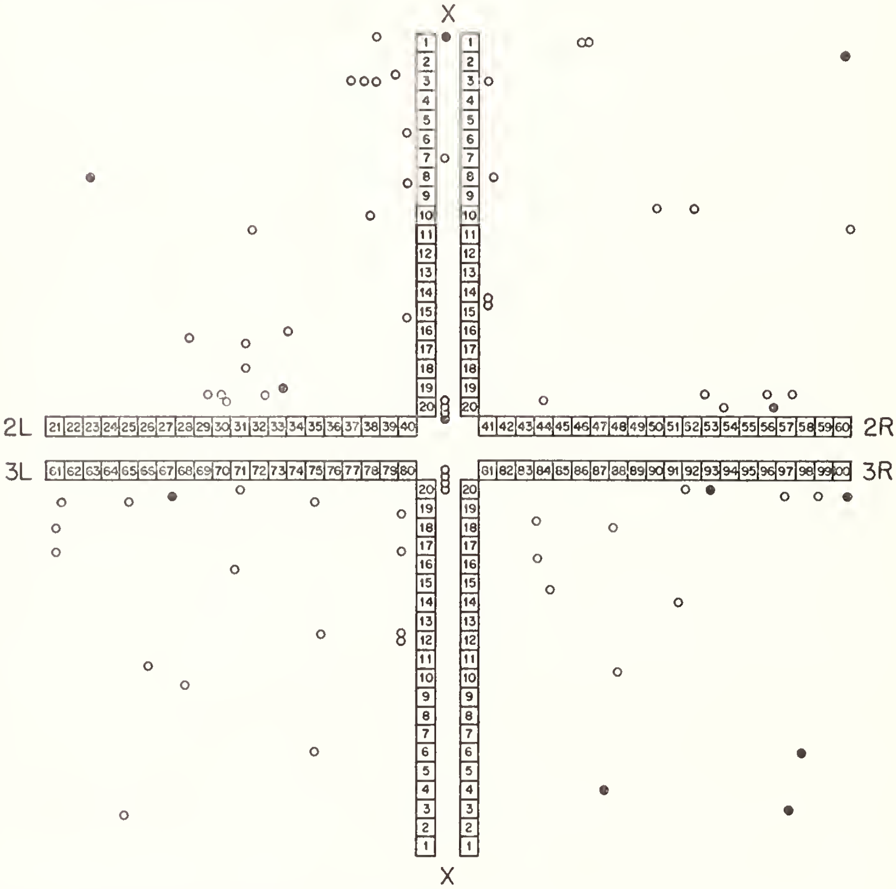
TABLE 5.—Characterization of X-ray-induced translocation between the X chromosome and chromosomes two or three

	Experiment	Lethal	Sterile	Fertile	Total
T(1;2)	1959	3	5(2)*	7(5)	15
	1961	14	38(1)	7(1)	60
Total		17	43	14	75
T(1;3)	1959	2	5(2)	5(4)	12
	1961	8	37	6	51
Total		10	42	11	63
Total		27	85	25	137

* Numbers in parentheses represent apparent reversions of the original translocation; they are also included in the numbers to the *left* of the parentheses.

Of the 25 fertile translocations, 10 no longer show linkage between the X and an autosome. Those that were recovered in 1959 (*i.e.*, 9 of the 10) were retested shortly after recovery by examining linkage relations in males, and the presence of a translocation was confirmed. I believe that these translocations had a heterochromatic breakpoint in the X chromosome, and probably in the autosome as well, and they reverted through some type of heterochromatic exchange. The proximal region of the reverted X was not examined for evidence that such an exchange had taken place. Of the 15 fertile translocations that still show linkage between the X and the autosome, 2 are complex insertional translocations, 12 are simple reciprocal translocations, and 1 has not been analyzed.

Dr. Warters has completed the salivary analysis of the majority of these translocations; the breakpoints of the simple reciprocal translocations that are male viable are plotted in text-figure 2. Because of the bookkeeping problems associated with the large number of translocations involved in these studies there are still inconsistencies in the data; consequently, table 5 and text-figure 2 are still tentative, but the final tallies will not be greatly different. To the best of our knowledge, these represent an unbiased sample of T(X;A)'s, and so those viable translocations plotted on text-figure 2 may be confidently examined for a comparison of fertile and sterile translocations. Although the majority of the fertile translocations have their X chromosome breaks in the proximal heterochromatin (6 on text-fig. 2 plus the 10 reverted translocations, if indeed they are reversions) and could be thought of as being broken proximal to the region whose continuity is important to male fertility, 5 fertile translocations with euchromatic breakpoints are in direct conflict with X chromosome continuity hypothesis of male fertility. These 5 constitute grounds for abandoning the hypothesis, but the distribution of breakpoints of fertile versus sterile X;autosome translocations does not provide additional insights into the question. It seems quite likely that this is a phenomenon related to chromosome organization, but the solution of the problem will have to await further observations.



Text-figure 2.—Distribution of breakpoints of the viable reciprocal translocations between the X chromosome and chromosomes two and three. *Solid circles* represent male-fertile and *open circles* male-sterile T(X;A)'s. *Points* within the axes represent translocations with heterochromatic autosomal breakpoints in which the autosomal arm involved could not be ascertained.

RESUMEN

Los genes localizados en el mismo cromosoma no funcionan independientemente uno del otro. Entre los genes hay relaciones espaciales que juegan un rol importante en la acción génica. Evidencias en favor de esta aseveración se los halla en tres tipos generales de observaciones:

1) *Actividad coordinada:* Genes que están funcionalmente relacionados pueden presentarse situados cerca de los otros sobre el cromosoma formando una agrupación conocida con el nombre de operon el cual es controlado por un solo mecanismo.

Genes que están sobre el mismo cromosoma pueden ser controlados coordinadamente por la respuesta de todo el cromosoma a mecanismos que hacen que el mismo resulte heterocromático o eucromático o que esté presente o ausente en diferentes células. Puede inferirse en el último caso que, debido a estar coordinadamente controlados, los genes de estos cromosomas pueden estar funcionalmente relacionados.

2) *Efecto de posición:* Un gen en su posición normal puede funcionar a distintos niveles en dependencia de la constitución genética de otra región del mismo cromosoma; esta región puede estar inmediatamente adyacente o a alguna distancia del gen en cuestión. Se sabe también que los genes funcionan anormalmente cuando son transferidos a regiones cromosómicas anormales.

3) *Continuidad cromosómica:* Se sabe que ciertas actividades cromosómicas, sino génicas, dependen de la continuidad lineal del cromosoma entre regiones cromosómicas específicas y que, la interrupción de esta continuidad por rearreglos o recombinación, interfiere con la función normal.

Estas clases de fenómenos están relacionados entre sí. En el texto se citan ejemplos de cada tipo.

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DISCUSSION

Cordeiro: I am not sure if you presented evidence that there is no change in the chromosomes in the point that translocation occurs. Can you be sure there is nothing lacking?

Lindsley: I can argue that alteration of the action of a gene near the point of breakage is not responsible for male sterility, since sterility is associated exclusively with reciprocal translocation and not with other kinds of rearrangements. Any loss of material or position effect that one might postulate should be associated with inversions and insertional translocations as well as reciprocal translocations.

Schultz: I might perhaps at this point respond to Dr. Lindsley's comment about the position effects exerted by the heterochromatic regions. Before doing so, I should like to make a point about the use of the term "heterochromatin." Dr. Cooper said that he was perhaps the only person in the world who did not believe in heterochromatin. He is not quite so isolated as that. It has been evident for a long time that the terminology was loose and confused. Functionally inactive chromosome regions tend to be compacted, and the term heterochromatin was devised by Heitz for those special regions of the chromosomes which assume this state in most mitotic interphases. But the use of such a term implies a genetic identity between the regions, and this seems not to be the case. Yet they do have in common a delayed replication cycle, and a deficiency of RNA synthesis. The situation in *Drosophila* particularly has been clarified by the work in our laboratory. The heterochromatic regions of the mitotic metaphase chromosomes comprise between a fifth and a third of the total DNA of the complement; yet in the giant salivary gland chromosomes, this is reduced to about a thirtieth. By measurements of DNA during the early development of the salivary gland nuclei, Rudkin has now provided evidence that this difference is due to a failure of replication in these regions—part of the differentiation of the salivary gland nucleus.

In the rearrangements which show variegation, the position effect of the heterochromatic regions affects the activity of the transposed locus, usually diminishing it. A tenable hypothesis for this phenomenon is the imposition on the new neighbors of the replication and activity characteristics of the heterochromatic regions to which the transposition has taken place. On such a basis, the cytology of the giant chromosomes in these rearrangements should show parallels with the variegation—a thesis for which there is evidence, as well as some controversy. With the diagnosis of the puffing of the giant chromosomes as the evidence of gene activity, the opportunity to re-examine the earlier cases in terms of the cycle of activity in these chromosome regions was undertaken. In the best studied case the rearrangement occurred just at a region (3D of the X chromosome) in which puffing occurs. It turns out that the rearranged fragment does not puff when the normal homolog does except under circumstances (environmental or otherwise) when the variegation is suppressed. Thus, the correlation exists between the manifestation of activity at the chromosomal level and the somatic variegational effects of the genes in the same region.

The control of replication and of function in the chromosome is presumably mediated by the extension and compaction of the DNA template. The heterochromatic regions proper in their position effects exhibit a controlling function on the genes placed next to them in the rearrangements by way of their cycle of compaction which influences the compaction and extension cycle of the loci placed next to them. These, with the new compaction cycle, do not function in their normal way; hence, they give the variegational position effects that we have seen. I assume this problem of the compaction of the template to its function will occupy us at a later session.

Cooper: I'd like to ask Dr. Lindsley whether he re-examined the chromosomes in the spermatogonia of meiotic cells. Do you know that everything is there at the beginning of spermatogenesis in those sterile translocations with autosomal involvement?

Lindsley: Yes, I see all the chromosomes in the primary spermatocytes.

Cooper: Is there a loss of material during spermatogenesis?

Lindsley: No.

Giles: I wish to ask Dr. Lindsley one rather general question: To his knowledge is there any general difference between the expression of variegated position effects involving inversions on the one hand and reciprocal translocations on the other?

Lindsley: No.

Schultz: I should perhaps add there are differences between different rearrangements but they do not fall into that category. The important factor is which locus is placed next to which, and there is no special relation to orientation with respect to the centromere.

Giles: My question was based on the possibility that there might be a difference by virtue of the changed polarity with respect to the two situations, but apparently this is not the case.

Pavan: Looking at your last figure, Dr. Lindsley, I did not notice whether the translocation breaks are randomly distributed or whether they tend to occur in certain portions of the chromosome.

Lindsley: The translocation distribution as it was plotted seems to indicate a clustering of breakpoints toward the proximal ends of chromosome arms. I think this is an artifact of using salivary chromosome length as the coordinates. Had the breaks been plotted as a function of mitotic or breakage length, then I believe the distribution would have appeared to be random.

Wolff: For clarification, I would like to ask Dr. Giles what type of polarity he referred to: the polarity that one might expect for gene action if the genes are in different order, or is he thinking in terms of the strands of DNA becoming inverted and then rejoining with one another so that the code is messed up because only one strand is read.

Giles: I had in mind the question of polarity primarily at the molecular level, particularly in view of the suggestion that inversions in prokaryotes should behave as deletions if only a single strand of DNA is read.

Wolff: But again, since inversions can occur in higher forms, I think this is additional evidence that the chromosome in a higher form is complex and not just a single DNA molecule. It might even indicate that the chromosome has material other than DNA contributing to its linear continuity.

Schultz: I doubt that one can easily come to conclusions about the continuity of DNA on the basis of chromosome behavior. For example, to initiate a replicating unit, a DNA sequence allowing the function of a specific polymerase might be required. The logic that says that because one has a discontinuity in function there need necessarily be a discontinuity in kind of substance seems a little dangerous.

Wolff: I think the arguments on inversions with a single DNA molecule maintain that when a piece of DNA with two different strands, each running in opposite directions, is taken out and inverted, it has to be twisted to maintain the 3 to 5 polarity. If you read only one strand of the DNA molecule, as the evidence seems to indicate, when you reach the point of inversion you switch off and start reading the codon from the reciprocal and inverted strand, which should result in genetic nonsense. This could preclude the formation of viable inversions.

Schultz: I agree with that, but one can imagine other mechanisms than those involving protein linkers, although I should emphasize that there are no data to exclude those possibilities so far as I know.

Chromosome Metabolism

Chromosome Functions in Specific Regions [1]

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IN THIS presentation I should like to touch on two aspects of chromosomal function. The first is the puffing phenomenon in giant chromosomes. The second is the phenomenon of variegation in the eye of *Drosophila.*

GIANT CHROMOSOMES

Several tissues of dipterous insects possess giant chromosomes. It is now generally accepted that these chromosomes are the result of several, in some cases as many as 13, replications of a single stranded chromosome during larval life. They have a ropelike structure of up to several thousand strands. The banding pattern is believed to be due to differential coiling of each strand. According to this interpretation a single band is the sum of the coils at any one section.

Recent autoradiographic and microspectrophotometric studies in *Chironomus* performed by Keyl and Pelling (*1*, *2*) suggest that the whole chromosome begins with its replication simultaneously. The time needed to complete replication increases with the thickness of the particular band; heterochromatic regions need the longest time.

Whether the deoxyribonucleic acid (DNA) goes as a continuous strand through the whole length of the chromosome or whether it is confined to the banded regions only, as claimed by Steffensen (*3*), is still a matter of controversy.

The time of 20 hours calculated by Keyl and Pelling for the last replication of the whole chromosome seems longer than might be expected for *Drosophila.* In *Drosophila* a replication could, on the average, take only about 10 hours since there is only a time span of about 100 hours available for 10 replications. If, as in *Chironomus*, the last cycle of replication would take 20 hours, then earlier cycles would have to be much shorter. That would mean a dependence of replication time on chromosome size of about this order: If the first polytenization step would take 1 hour, the next one would take 3 and so on, *i.e.*, 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 hours. With such a scheme we have 10 cycles of replication, the last taking about 20 hours and the sum of all 10 about 100 hours.

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 5, 1964.

From the information available on the rates of cell division in other tissues in *Drosophila, e.g.*, the eye anlage (*4*), there seems no basis for the assumption that the replication of a single-stranded chromosome would take one twentieth of the time of a 512-stranded chromosome. That is, one would expect the rate of replication for a single strand would be nearer a constant, whether it was in a cell with a normal set of chromosomes or in one with a polytene set of chromosomes, since the ratio of nuclear size to cell size appears constant. However, these relationships are still to be investigated.

Puffing

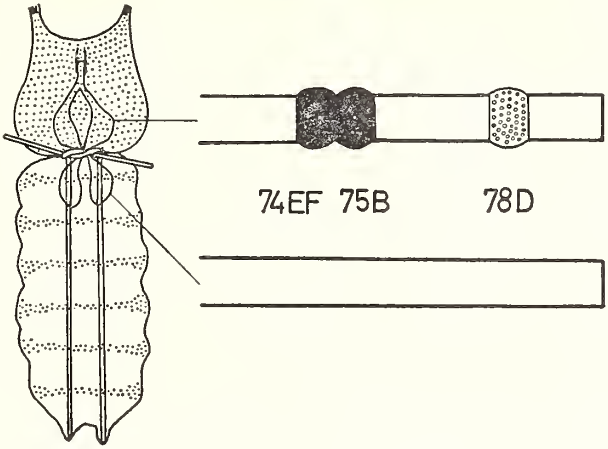
Certain regions of the giant chromosomes do not always show the same clear banding pattern, but are occasionally puffed (fig. 1). Puffing is believed to go along with the uncoiling of one or more bands. The bulk of the material accumulated within a puff is ribonucleic acid (RNA) and protein. The RNA is most probably synthesized *in situ,* more in bigger puffs and less in smaller puffs. Pelling (*5*) found close to 200 puffed regions within the chromosomes of a single salivary gland cell. Although in the salivary gland cells of various animals, different regions can be found in a puffed state—277 such regions have been identified in the salivary glands of *Chironomus*—the puffing sites are in general characteristic for each type of tissue in which giant chromosomes are found. This is understood as a sign of differential activity in the different types of cells.

The proteins accumulated in a puff do not seem to be synthesized at the puffing site, according to Clever (*6*) and Ritossa *et al.* (*7*). The origin and function of these proteins are as yet unknown.

The elegant microelectrophoretic analysis by Edström and Beermann (*8*) indicates that the types of RNA synthesized by different chromosome regions vary in their base composition. This suggests that messenger RNA is produced in the puffs. Only the nucleolar RNA shows a base composition similar to that of the cytoplasmic RNA, which supports findings in other material that ribosomal RNA is produced by the nucleolar organizer.

Puff Induction

Many puffs seem to be formed at more or less irregular intervals, as Clever (*9*) has shown. The RNA of these puffs probably controls the synthesis of enzymes of the cell's basic metabolism. In addition there are other puffs whose appearance shows a close correlation with the molt cycles during larval development. Some of them owe their appearance to the induction by the molting hormone (ecdysone), which is produced by the prothoracic glands. An easy way to show this is by the ligation of *Drosophila* larvae (*10*) (text-fig. 1). A ligature is placed around the larva so that the salivary glands are separated into about equal halves.



Text-figure 1.—Larva ligated before hormone production has formed a puparium only anteriorly; only in this part of the larva have the salivary gland puffs in the proximal portion of chromosome III been formed.

Then the anterior portion of the animal will contain the hormone-producing ring gland. If the animal is ligated before hormone production, only the chromosomes in the anterior halves of the glands will develop the puffs characteristic for the prepuparial hours.

The work of Kroeger (*11*) and Clever (*6*) indicates that the hormone can induce several puffs independently. Only two of the *Chironomus* puffs, however, seem to react exclusively on changes in hormone concentration. The threshold for the reaction of one of them is at a concentration of about 10^{-7} μg ecdysone per mg of larval weight, which is supposed to correspond to about 10 molecules of ecdysone per haploid set of chromatids.

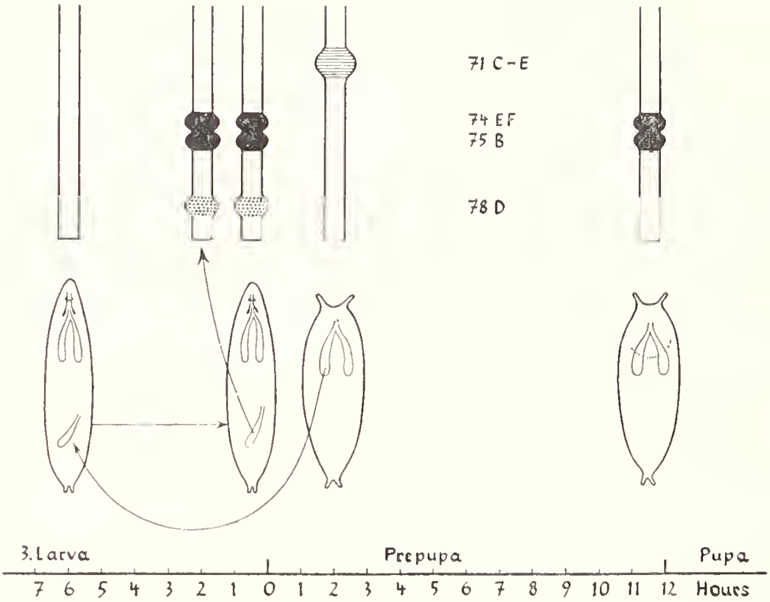
One observes changes of puffing patterns from one developmental stage to the next. These are at least not generally due to changes in reactivity of the loci concerned, as we showed by transplanting glands (*10*) (text-fig. 2). The reversibility of the puff in region 78 D indicates that not the puffing site itself but rather some inducing agent other than ecdysone changes from one molt to the next.

Treatment with juvenile hormone (*12, 13*), which is produced by the corpora allata and determines the character of a molt, has not directly affected chromosomes as yet. Also the pattern of puffs primarily induced by ecdysone is identical at both larval and pupal molts. It is deduced, therefore, that the interaction of the juvenile hormone with the effects of ecdysone lies on the level of more secondary processes in cell metabolism.

Whether the action of ecdysone on the puffing sites is direct or via a primary effect on the cellular ion pump, as Kroeger (*14*) insists, is still under discussion.

Genetics of Puffs

Strong efforts are being made to find genetic differences in puffing behavior. In one most favorable example, in which Beermann (*15*) com-



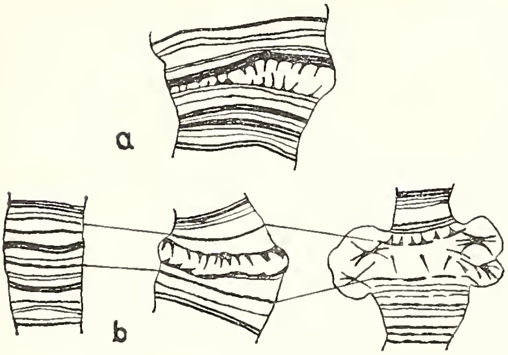
TEXT-FIGURE 2.—The host larva with gland implanted from 2-hour prepupa is sacrificed when it approaches the time of puparium formation. The puff in region 78 D of chromosome III is formed in both host and implant glands. The prepupal chromosomes do not normally form this puff.

pared and crossed two species of *Chironomus*, the presence and absence of a puff are correlated with the presence and absence of secretion granules within the cell type, for which both puff and granules are characteristic. The presence of the granules is like a semidominant trait. In the species' hybrids, only half the usual number of granules are present, and the respective puff is formed by only one of the two homologous chromosomes.

Another case is the recessive mutation *fat* in *Drosophila,* which makes carriers develop a stout shape of body and wings. The gene has long ago been localized in the region 24 D/E on the cytological map of the salivary gland chromosomes. Slizynski (*16*) found that larvae carrying the mutant *fat* allele produce vacuoles in the posterior cells of their salivary glands and along with it a puff in the chromosome region corresponding to the site of the gene, *i.e.*, 24 D/E. The anterior gland cells contain neither the vacuoles nor the puff.

Otherwise only the absence of whole sets of puffs has been reported, by us (*17*) in the mutant *lethal-giant-larvae,* in which the larvae die shortly before or after puparium formation, and recently by Rodman and Kopac (*18*) in the mutant *tumorous-head,* whose larval life is somewhat prolonged.

Heterozygosities of puffs can be of two different types (text-fig. 3): Either the puff is present in one and absent in the other one of the two homologous strands, as has been demonstrated in *Chironomus* (*19*), or the heterozygote shows an intermediate-sized puff throughout both homologous



Text-figure 3.—(*a*) Section from salivary gland chromosome of *Chironomus* with heterozygous puff (after Beermann). (*b*) Homologous sections of chromosomes from anterior lobe of *Acricotopus* salivary gland, with standard type (*left*), inversion type (*right*), and hybrid (*middle*) (after Mechelke).

strands as was found by Mechelke in *Acricotopus* (*20*). The basis for this difference is not understood.

To gain more insight into this very suitable phenomenon for the study of gene activity and its control, the search goes on for more tissues carrying giant chromosomes and for means to increase the size of available chromosomes. Whitten (*21*) has described well-analyzable giant chromosomes in the footpad of *Sarcophaga*. In salivary glands of *Drosophila* larvae implanted into adult flies, Hadorn *et al.* (*22*) found that the chromosomes obviously kept replicating and developed to "super-giant" size. According to Matuszewski (*23*) even parasitizing hymenopteran larvae will induce the cecidomyid fly *Dasyneura urticae* to develop a giant chromosome containing tissue around the intruder.

Another interesting problem came up with the observation that at the tip of chromosome I in *Drosophila melanogaster* 3 puffs, located in linear order 1–2–3 on the chromosome, appear and disappear in that same order (*17*). This happens in both puffing phases, the end of the last larval instar, and the end of the prepupal stage, each time during an interval of about 4 hours. We are presently investigating the behavior of these puffs in a complex translocation stock kindly provided by Dr. Lindsley, in which one of the breaks has separated puff 1 from puffs 2 and 3 and all 3 are now attached to the third chromosome centromere. We want to find out whether the normal puffing of puffs 2 and 3 depends on their being closely linked to puff 1 as in the intact chromosome. Preliminary inspection of the chromosomes suggests that it does, but more accurate quantitative measurements are needed.

VARIEGATION

Somatic

We are also interested presently in some functional aspects of this same chromosome region. This region contains two genes involved in the control of the synthesis of eye pigments. They are *zeste* (*z*), located in band 3A3, and *white* (*w*), located in 3C2. Twelve bands lie between them;

yet they show some close functional relationship: $\frac{zw^+}{zw^+}$ flies show *z* phenotype, *i.e.*, orange eye color instead of the normal red, and $\frac{zw^+}{zw}$ show normal red phenotype; this latter phenotype is due to the presence of only one w^+ allele, as Gans (*24*) has conclusively shown.

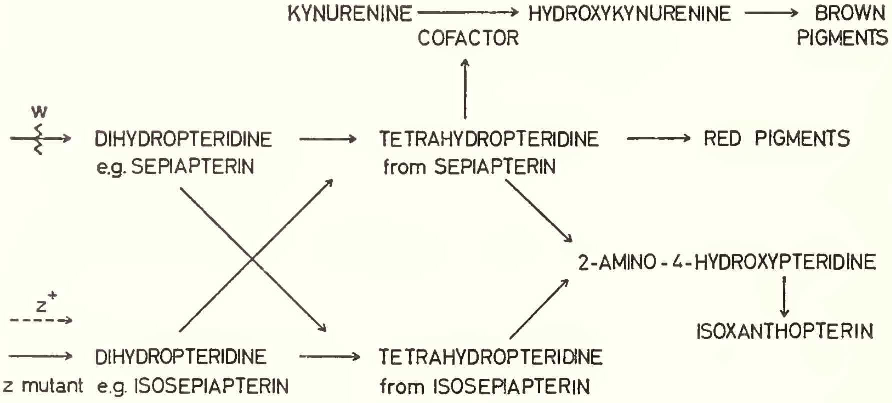
The interpretation that H. Forrest presented (personal communication) is shown in text-figure 4.

Two doses of *zeste* mutant result in an overproduction of isosepiapterin, which inhibits the reaction above: sepiapterin→tetrahydropteridine. No red pigments will be formed, but there is enough leakiness to give some tetrahydropteridine used as cofactor for the production of brown pigments, that is, the *zeste* phenotype.

With *zeste* homozygous + *zeste* suppressor (*i.e.*, a *white* allele) sepiapterin production is lowered; thereby the inhibition of the reaction isosepiapterin→tetrahydropteridine is relieved (leading to subsequent formation of 2-amino-4-hydroxypteridine and isoxanthopterin). Consequently, the concentration of isosepiapterin is lowered, which, in turn, relieves the inhibition of the reaction sepiapterin→tetrahydropteridine, leading in the end to both red and brown pigment formation.

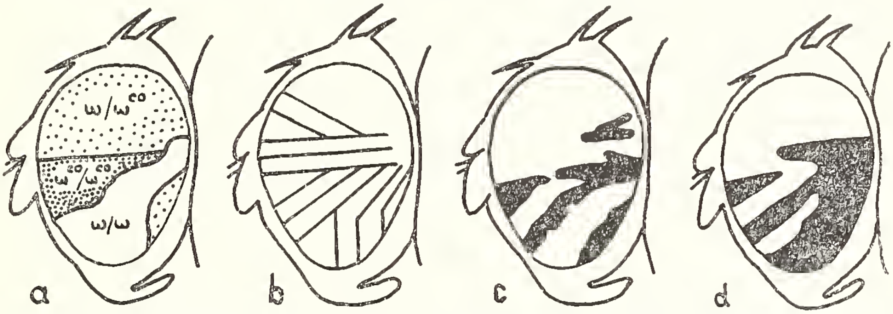
In agreement with this interpretation, those members of the *white* allele series, which are the suppressors of *zeste*, do not show any dosage compensation. According to Judd (*25*), a small duplication of the sublocus carrying these alleles will lead to a *zeste* phenotype in males (it is the sublocus No. 4, carrying *white-cherry*, several *eosin* alleles, and *white-honey;* however, the latter does not fit into the scheme). Since in such a case the mutant *z* allele is present only in single dose, *zeste* itself appears to be dose-compensating.

Our main interest is in one type of *zeste* that supposedly has a small duplication of sublocus 4 in the *white* region. Before this was known, the mutant was called z^l (*zeste-light*) since the males were not normal


KYNURENINE
HYDROXYKYNURENINE
BROWN PIGMENTS
COFACTOR
W
DIHYDROPTERIDINE e.g. SEPIAPTERIN
TETRAHYDROPTERIDINE from SEPIAPTERIN
RED PIGMENTS
2-AMINO-4-HYDROXYPTERIDINE
ISOXANTHOPTERIN
z+
z mutant
DIHYDROPTERIDINE e.g. ISOSEPIAPTERIN
TETRAHYDROPTERIDINE from ISOSEPIAPTERIN

TEXT-FIGURE 4.—The interaction of the genes *white* (*w*) and *zeste* (*z*) on the formation of eye pigments in *Drosophila melanogaster.* For details *see* text (after Forrest).

in phenotype but basically *zeste*, but with patches of red areas in the eyes (*26*) (text-fig. 5*c*). The origin of these patches is partially explained by earlier investigations, in which similar patches were induced experimentally (*4*). A twin spot (text-fig. 5*a*) arose after a somatic crossing-over in the X chromosome was induced by X rays in a young anlage cell of a w/w^{co}; se/se larva. The sum of many such spots yielded a pattern (text-fig. 5*b*). Each of the sectors is formed by one anlage cell from the end of the first larval instar after about 10 cell divisions during the subsequent larval and pupal life. What they have in common with the patches in z^l males is their shape. In the z^l mottling, therefore, each of the dark sectors is also derived from a single anlage cell.



TEXT-FIGURE 5.—Left eyes of *Drosophila melanogaster* with (*a*) X-ray-induced twin spot, (*b*) pattern of eye sectors formed by cells from first larval instar eye anlage, (*c*) eye of a z^l male with red patches (*black*) on lemon-colored background, and (*d*) eye of translocation-heterozygous female ($w/w^{258\text{-}18}$) with dark-red patches (*black*) on lighter background.

The rather uniform size of the spots in z^l indicates that at a definite stage in development a decision is made independently for each single cell in the eye anlage as to whether both subloci 4 will become active or not. From the time of decision to the midpupal stage, when activity sets in, about 10 cell divisions take place. Over the time interval of these 10 cell generations, the capacity of one cell family and the incapacity of the adjacent cell family to become active at the end of this period are stably inherited.

We have also been able to furnish the following evidence that the property of a cell is really located in this particular chromosome region. z^a Animals have entirely normal eyes. The mutant character shows up only in compounds with other *zeste* alleles, for instance $z^l.z^a/z^l$ animals have *zeste* eyes with occasional red mosaic spots. Within the *zeste*-colored background of the eye, one can restore the pigment-forming capability by inducing somatic crossing-over in the X chromosome and thereby making a cell homozygous for z^a. From this we conclude that the fixation of the ability to form pigment happens on the chromosome, most probably on the *white* locus. Presently we are performing an experiment to corroborate this conclusion.

Another similar phenomenon has been observed. In this case there is also the *white* locus of the X chromosome involved and, furthermore, these flies show also mosaic spots identical to those resulting from radiation-induced somatic crossing-over (text-fig. 5*d*). Even the size of the mosaic spots is the same as those in the z^l males.

In this case the origins of mosaicism are structural changes in the chromosomes, in our particular case a transposition of the normal *white* allele from its normal location into a heterochromatic chromosome region. Schultz (*28*) has observed that heterochromatin can heterochromatize neighboring loci. He has also shown that the heterochromatization of a euchromatic locus depends on its distance from heterochromatin and, within a single cell, on chance. The closer to heterochromatin the greater the chance for a locus to be heterochromatized.

In these mutants the euchromatic *white* locus remains functional in one cell and red pigment is formed, whereas in the adjacent cell the *white* locus becomes heterochromatized, a condition that is inherited over the following 10 cell generations and which leads to the formation of a light sector in the eye. The setting in of heterochromatization during definite stages in development and the stability of a once achieved state, euchromatic or heterochromatic, are known, *e.g.*, in insects (mealy bugs) (*29*) and mammals (*30*).

In both the *zeste* and *white* heterochromatin cases, we are dealing with 1) the establishment of two alternative functional states of chromosome loci, at 2) a characteristic time during development, which shows 3) a remarkable stability during all subsequent cell generations.

A definition of the genetic basis of cell differentiation could probably be rather similar to the sentence just given (*31*). Since we are interested in the fixation of functional capabilities of cells at characteristic times during development, we ask ourselves: What is the difference between the *white* locus in one z^l cell and the *white* locus in an adjacent cell, or, in the other case, between a euchromatic *white* locus in one cell and the heterochromatized *white* locus in its neighboring cell?

Germline

Since so far it has been difficult to propagate single somatic cells of insects, we are grateful that we may have a similar type of mosaicism in cells propagating naturally, that is, in the germline.

We have found in our *zeste* stock a light-eyed male. This light eye color was connected with an inversion which arose spontaneously in the X chromosome. The left break lay, as far as we could see, between 3C1 and 3C2, *i.e.*, just to the left of the *white* locus, and the right break lay in the heterochromatic region of the chromosome.

We know that whenever the normal function of the *white* locus is impaired the pigment production is reduced. But we also learned above that an impairment of *white* shifts *zeste* phenotypes toward normal. We can, therefore, expect from a constitution, *zeste* plus an inversion of the mentioned type, almost any phenotype between normal and *white.* And that is what we found in the male offspring after we crossed it to attached-X females.

The amazing thing is that some flies had only very dark eyes, others only very light ones, and that, although all eyes of this new stock showed a mottling effect, the two eyes of one fly were rather uniform. This seems to indicate that the phenotype of a fly is already determined in the germline of the preceding generation.

Unfortunately, this type of germline variegation, as we suppose it might be, is rather difficult to distinguish from segregation of genetic units. Theoretically, the occurrence of light and dark offspring from a single light fly can also be explained on the basis of a simple dominant enhancer of variegation. The original cross would look like this:

$$\mathrm{P}:\ +/+ \times \mathrm{En}/+$$

$$\mathrm{F}_1:\ +/+ \text{ and } \mathrm{En}/+$$

The F_1 generation would show light and dark flies in about equal proportions. And we have found that dark-eyed animals have only dark-eyed offspring.

Some observations we made, however, do not agree with the assumption that the phenomenon is due to a simple enhancer of variegation: 1) Among the offspring of light-eyed animals, there were more types with different degrees of pigmentation than can be expected on the basis of a single enhancer gene; 2) we have been unable so far to localize whatever is responsible for the decrease in pigment on any one of the chromosomes.

Another hope that we are dealing with a germline variegation is a second case we found recently. In our *sc* z^m stock, which normally has maroon-mottled males, some males had a phenotype typical for ordinary *zeste* females, *i.e.*, lemon-colored eyes. Crossed with attached-X females, the male offspring showed, as expected, the same phenotype as their fathers. But among them were regularly a few exceptions with phenotypes like *z* males, *i.e.*, entirely normal pigmentation. Such few exceptions do not seem to indicate that a dominant enhancer is involved.

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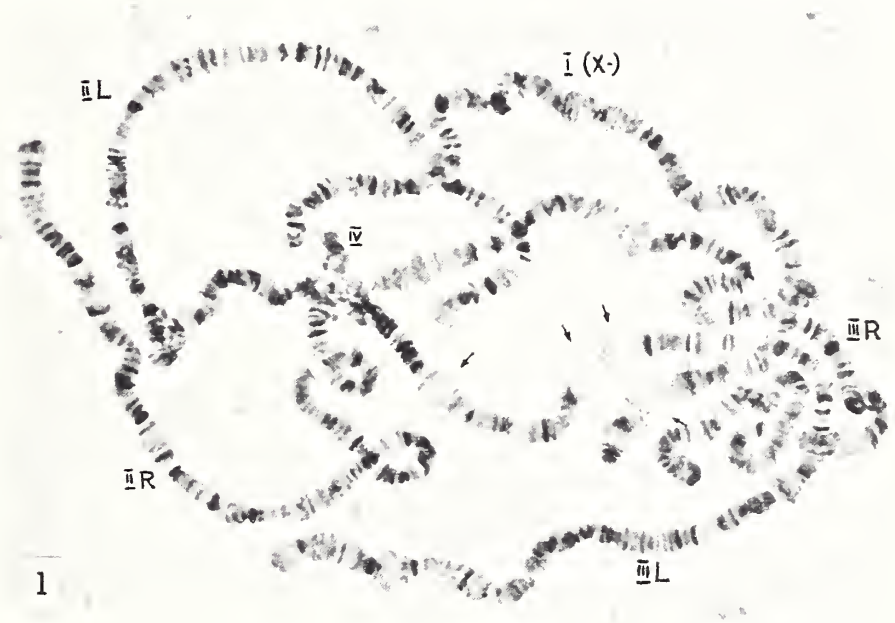

FIGURE 1.—Salivary gland chromosomes of *Drosophila melanogaster* with puffs, especially at the basis of III L and at the distal end of I (*arrows*).

DISCUSSION

Moses: So far we have tended to look at the chromosome as if its sole function were to sequester, to replicate, and to transmit genetic information to future generations. These are very beautiful examples of the other aspect of chromosome function, namely, the transcription and translation of information into metabolic events.

Cordeiro: You showed two types of heterozygosity in puffing. One works as expected, which is that one of the two homologous chromosomes shows the puff. But another example you gave shows a type of intermediate puffing. Do you explain this intermediate puffing as the influence of the action of a gene, or simply that it is a mixed strand which you have superimposed morphologically?

Becker: There is certainly no mixing of the two parental strands in giant chromosomes. This seems to be a genuine intermediate expression of a puff in both strands.

Reissig: Would not that sort of heterozygosity fit very nicely the structural mutation on the one hand and suppressor mutation on the other?

Becker: On the basis of the regulator gene—operon system one type could be explained, I suppose, as the mutation of a structural gene or as an o^c-mutation, the other, intermediate type as an i^s-mutation of a regulator gene.

Lindsley: I would like to ask with respect to this *zeste* inversion whether there is any difference in the expression when the inverted chromosome is inherited maternally and when it is inherited paternally.

Becker: There is no difference.

Hoffschneider: Do you know of any experiments proving that the hormones act directly on the genes or whether there are intermediate compounds which could be used by the hormones indirectly on the genes?

Becker: The only experiment I am aware of is that of Clever, who insists that the hormone acts directly on the chromosome. Kroeger, on the other hand, had reason to discuss the possibility that the hormone influences primarily the cellular ion pump, *i.e.*, the influx and outflux of sodium and potassium ions. Clever has tried to repeat Kroeger's experiments but, as far as I know, without success.

Cooper: Do I understand you correctly? You think there are germline changes, determined in the preceding meiosis, that are responsible for the bilateral patterns you find in the eyes?

Becker: So far it seems that way to me; that is, in germ lines there are changes, each of which determines the phenotype of an individual among the offspring.

Cooper: You know that a variegation is brought about in wild-type flies simply by the addition of Y chromosomes. In these cases the Y chromosomes may be introduced either from the mother or from the father, and nevertheless there is a tendency for a symmetrical pattern in the two eyes whenever the variegation is extreme; so, I would question then whether the evidence of pattern reflects importantly upon germline changes. I think of this effect of Y chromosomes as a position effect that is produced without any change in position, because you can imagine that the way in which a gene or a locus variegates is dependent upon a change in either its time for action or its presence during the existence of the stage that is necessary for its action. Suppose we have some substrate that is necessary for a gene to produce its effect, and we simply have a beginning and an end to the state in which this substrate is present or a time period in which a gene might act. If this were the case, most genes that give self-coloration or uniform action would tend to fall with their action at the midpoint of this state. Now, in the case of position effect, we can imagine that the new environment of the gene essentially puts it at one end or another near a terminus of its state. In this event, the genes may or may not act because there is variance to its activities.

A gene that is moved totally beyond the limits of the state that it requires again gives a self-colored phenotype. Now, the addition of Y chromosomes to w^{m4}, for example, has the following property. The w^{m4} gene in the presence of a single Y variegates; add another Y and with the two Y chromosomes, it is almost a wild type. But, in the 3Y condition, once again phenotypic variegation occurs and in this case the variegation is probably due to genes that are not necessarily associated with the Y locus. This is because the additional Y chromosomes somehow create a relationship such that the total amount of heterochromatic elements to euchromatic elements is widely changed. Conceivably the state in which some genes act is changed, and hence variegation of some wild-type genes may be produced simply by adding Y chromosomes. Conversely one ought to be able to produce variegation by reducing the Y chromosomes. But the fact that they tend to produce bilaterally symmetrical variegation makes it unlikely that the determination of this bilateral event can occur early in the germline.

Becker: I agree with you that bilateral symmetry of pigmentation itself is no evidence for changes in the germ line. The point is, that there is hardly a difference between the pigmentation of the two eyes of a single fly, but very strong differences between the darkest, the lightest, and all the intermediately pigmented flies within one sibship.

Cooper: Dr. Becker, if you collect all females that have two Y chromosomes, you will find that variegation in them also varies, and this is also true if 3 Y's are present. Consequently, while these observations of yours are consistent with your notion, I do not think that they are compelling and force one to agree with you.

Becker: Again you are right, but preliminary examination has not shown any extra Y chromosomes to be present in this stock and, in addition, the variety of pigmentation types within one sibship is greater, I should say, than one could expect simply on the basis of extra Y chromosomes segregating. But further investigation is certainly needed.

Schultz: I would like to ask where the locus of the inversion is, particularly with respect to *zeste.* More specifically in the inversion which you described, you gave the possibilities for variegation as 2 separate loci which are interacting; this can be complicated, in my own experience. With a variety of loci one can get quite confusing interactions of this kind. It is quite conceivable that the variations you observed could be due to a determination in each individual of one or the other locus first which would change the mean of variegation in a quite definite way. From what you have described I think it would be amusing to work it out. I wonder whether a possible approach to this might not be to find out precisely what the interaction of this heterochromatic region transposition is with those at the *white* locus; one could make such stocks. With regard to the germline, have you studied the effects of brooding the males? Is there any age difference in the type of thing you find? What I have in mind, of course, is the sort of paternal effect that Spofford described in the *white* variegation, which could look like such a germline change, particularly if you had such a factor segregating. Have you tested the effect of age on the variance of your variegation patterns?

Becker: In answer to your first question, the break seems to lie at the same place as the break in w^{m4}, that is, between 3C1 and 3C2, bringing the 3C2 band closer to heterochromatin. With respect to the second question, this is indeed what I expected too, and so I tried to find a brood pattern. There is none. The distribution of different phenotypes among the offspring from single males and females is uniform throughout all the brooding ages.

Chromosomal Differentiation [1, 2, 3]

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SPECIFIC CHANGES occur in the nucleus as well as in the cytoplasm of cells during the process of differentiation. Until recently, it was generally believed that, except for the rare and specific cases in which duplication and/or reduction of genetic material occur (Mather, 1948), nuclear changes during differentiation were only of physiological order and always completely reversible. Briggs and King (1959), Fischberg *et al.* (1963), and Gurdon (1963), using the method of transplanting nuclei of several types of differentiated cells to anucleated egg cells, showed that the problem of irreversible nuclear changes during differentiation is probably more common than it was supposed. In experiments with *Rana*, stable changes in the nuclei of the cells were found to occur in differentiated cells, including some in the endoderm layer. When the nuclei of these cells were implanted in anucleated egg cells, they gave rise to abnormal embryos, some with specific syndromes that were repeatable upon reimplantation. Some of these nuclei were associated with chromosomal abnormalities which were believed to come from the process of transplanting the nucleus, but in other cases the nuclei showed, as much as one can see by the usual cytological methods, a normal set of chromosomes. From the findings of those studying the development of microorganisms, this nuclear differentiation is commonly considered as resulting from processes of inactivation of certain genes. This inactivation would cause metabolic deficiencies that would induce abnormal development of the embryos (Jacob and Monod, 1961; Monod and Jacob, 1961; Becker, 1964).

On the basis of our work on the polytene chromosomes of *Rhynchosciara angelae*, I will suggest another possible explanation for the origin of the irreversible nuclear changes that in association with the theory of the inactivation of genes may help to clarify the complicated problem of cellular differentiation.

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[2] Some of these studies were supported by the National Institutes of Health (contract GM 11493-01), Public Health Service, and by Fundacão de Amparo a Pesquiza do Estado de São Paulo (FAPESP).

[3] This paper is dedicated to Professor H. Bauer in honor of what he is doing and has been in his 60 years of life.

[4] I wish to thank Mr. David Amundson for his excellent editorial assistance and Mrs. Carmen Cumming for the translation of the Spanish summary.

NUCLEAR CHANGES DURING DIFFERENTIATION

According to Mather (1948), the known changes in the nucleus during differentiation are mainly: elimination, replication, and manifestation. I shall use the same classifications, and, in addition to the examples he gave, I will mention some new ones which I think are of interest in relation to the problem of nuclear changes. I hope to show also that corresponding changes can be found at the gene level.

The better-known examples of normal elimination of genetic material during differentiation are related to 1) the origin of somatic cells in *Ascaris* and in several species of Diptera and 2) the determination of sex in Sciaridae species. These have been discussed in detail by Wilson (1925), Metz (1938), White (1945), Mather (1948), and Stern (1958), among others.

The changes of replication, which would be produced mainly by endomitosis, would lead to polyploidy or polyteny. Polyploidy is caused by chromosomal division and separation without nuclear multiplication. It can occur in all or only in special cells of an organism, give rise to normal or abnormal cells or tissues, and even give origin to new races or species when it affects cells of the germline (White, 1945; Geitler, 1939; Suomalainen, 1950). Protozoa can give rise to differentiated nuclei of various types, as shown in the review by Grell (1964). Polyteny, on the other hand, is caused by multiplication of the chromosomes in a process by which the old and newly formed elements remain attached to each other and form a structure behaving as a morphological and physiological unit. The process is normal in the development of most tissue of the larva and the adult in the Diptera. The rate of polyteny is different in various tissues of an organism, normally reaching the maximum in the nuclei of the cells of the salivary gland of the larva. In several species of Diptera, polyploidy and polyteny sometimes occur together in cells of the same tissue. In the nurse cells of the ovary of *R. angelae*, Basile (unpublished) has shown that, besides an association of the processes of polyploidy and polyteny for certain chromosomes, condensation of specific chromosomes also occurs. These condensed chromosomes remain inactive during all the processes of development of the nurse cells, unlike the other chromosomes which appear to be very active during all the processes. In *R. angelae* the zygote has 11 chromosomes and an extra X chromosome, corresponding to the 5 pairs characteristic of the species (A, B, C, X, and the "limited"). At early stages of development, specific chromosomes are eliminated during the differentiation of certain tissues. In the development of the germline cells 1 extra X chromosome is eliminated, and the tissue develops with 10 chromosomes. During development of the somatic cells of the female the 2 "limited" chromosomes and 1 X chromosome are eliminated, and in the male, besides the 2 "limited" chromosomes, the 2 X chromosomes are eliminated. The germline cells of both sexes have 10 chromosomes, and the somatic cells have 8 in the females and 7 in the males. The nurse cells of the ovary of *R. angelae* derive from the cells of the germline, which retain the "limited" chromosomes of the species. At a certain stage of larval development, after several cycles of mitosis, each germline cell goes through a special division and will give rise to an oocyte and a nurse cell. These 2 cells remain attached until the maturation of the eggs. The nurse cell grows mainly during larva life, whereas the oocyte grows during pupation. Growth of the nurse cells is accompanied by a process of endomitosis in which all the chromosomes, except for the "limited," divide several times. The normal diploid number of germline cells is 10; at full growth each nurse cell has over 100 isolated chromosomes. Simultaneously with this polyploidy, a polytenic process also occurs in each of the dividing chromosomes in such a way that each isolated chromosome is in reality a bunch of many chromonemata. In this case certain chromosomes are duplicated, whereas others inside the same nucleus are condensed. I believe a similar situation can be shown in the cells of the salivary gland, but here the selected replication and condensation of genetic material are occurring at the gene level, as I shall discuss later.

The changes of manifestation are probably the most frequent observable in the nuclei of the cells of different tissues during the differentiation of any pluricellular organism. Striking changes of this type occur in the 2 daughter nuclei produced by the first division in pollen grains in plants (Mather, 1961), in the nucleus of cells of the mealybug (*see* Lindsley, this Symposium), and, of course, in the interesting condensation of 1 X chromosome in cells of tissues of the females of some mammalian species (Lyon, 1961; Grumbach *et al.*, 1963). In *Paramecium aurelia,* a unicellular protozoan, Sonneborn (1954) has shown that the micronuclei are practically inactive and have only reproductive function. The phenotype is determined by the macronucleus, which is polyploid and very active.

The work on polytene chromosomes of Chironomidae, Sciaridae, and Drosophilidae, in which bulbs, puffs, or similar structures have been observed, are, of course, typical cases of changes of manifestation at an intrachromosomal level (*see* Becker, this Symposium).

METABOLIC ACTIVITIES OF POLYTENE CHROMOSOMES

Several papers about the behavior of the polytene chromosomes of Diptera have demonstrated the importance of the data obtained in this chromosome for an understanding of the problems of gene activities and cellular differentiation. I shall not review these works, but will mention only specific cases in which extra synthesis of deoxyribonucleic acid (DNA) occurs in bands of polytene chromosomes and that are directly related to chromosome differentiation. For information about the importance of these changes for the clarification of problems of chromosomal physiology, I suggest some of the most recent papers: Becker, 1959, 1962; Beermann, 1956, 1959, 1963; Breuer and Pavan, 1955; Clever and Karlson, 1960; Gabrusewycz-Garcia, 1964; Gall, 1958; Kroeger, 1960, 1964; Pavan, 1958, 1963; Pelling, 1962, 1964; and Swift, 1962.

In the past 15 years many cytologists and geneticists have agreed, and still do, that all diploid nuclei of a given organism have the same amount of DNA. Others (for review *see* Brachet, 1957, and Vendrely, 1960) do not believe in this constancy of DNA. A strong argument both against the basis of this hypothesis and against its implications comes from the work of Breuer and Pavan (1955). This work was confirmed later by Ficq and Pavan (1957) and Rudkin and Corlette (1957), who showed that in *R. angelae* (Diptera, Sciaridae) certain bands of the polytene chromosome of the salivary gland cells have a disproportionate increase of DNA when compared with other bands. Similar results were obtained also by Swift (1962) and Gabrusewycz-Garcia (1964) in *Sciara coprophila.*

In *R. angelae,* due to its peculiar biological behavior of having groups of larvae all of the same age and sex and all individuals in the same stage of development, it is easy to follow the behavior of the polytene chromosomes of different organs during practically all stages of larval development. During the development of the larvae, certain bands of the polytene chromosomes of the salivary gland cells of *R. angelae* go through processes of localized swelling (puffs) in which extra quantities of ribonucleic acid (RNA), DNA, and, probably, protein are produced. During regression of the puffs, RNA is eliminated from the chromosome as micronucleoli and from the nuclear sap, where it stays for a short time before going to the cytoplasm. The DNA, forming a dense net of fibers at the puffed stage of certain loci, is subsequently condensed, determining the regression of the puff (Breuer and Pavan, 1955; Pavan and Breuer, 1955; Ficq, Pavan, and Brachet, 1958; Pavan, 1964; and unpublished data).

In some bands the extra amount of DNA produced before or after the puffed stage is such that the chromosomal section assumes an appearance typical of heterochromatin. But usually the amount of DNA produced is not very great, and after the puff regresses the band is only a little thicker than it was before.

Analyzing the polytene chromosomes of the salivary gland cells at several stages of larval development, using radioactive precursors and the autoradiographic method, one could show that this puffing behavior, sometimes with and sometimes without an extra synthesis of DNA, is not limited to a few bands; in different degrees it occurs in practically every band of the polytene chromosomes of *R. angelae.* During larval development each band, and in some instances groups of bands, of the polytene chromosomes of the salivary gland of *R. angelae* has its own behavior, which depends on the physiological stage of the gland and is normally different from the behavior of other bands located near or far from it. Apparently the only exceptions to this are the heterochromatic regions, which appear to act as a uniform system though distributed in different quantities at the centromeric regions of the different chromosomes. Chromosome B of *R. angelae* shows, besides the heterochromatin located near the centromeric region, another heterochromatic block located at the end of the first fifth of the chromosome starting from the base. This heterochromatic block normally reacts as part of the general heterochromatic system of the nucleus. On the other hand, the chromatic block with a heterochromatic aspect which is formed after the puffing process in some of the chromosomal loci, like the bands near the free end of chromosome B and C (shown in the work of Breuer and Pavan, 1955), behaves completely independently of the heterochromatin mentioned above.

The disproportional increase of DNA in specific bands of polytene chromosomes of the salivary gland of *R. angelae* shown by Breuer and Pavan (1955), Ficq and Pavan (1957), and Rudkin and Corlette (1957) was interpreted by some authors (Taylor, 1960; Patau and Das, 1961) as possibly being the result of asynchronous duplication of the DNA at different chromosomal regions. But this is not the case in *R. angelae*, because before showing this increase of extra DNA near their free ends, chromosomes B and C, for instance, passed through a series of cycles of DNA synthesis, and these chromosomal regions show particularly no changes during 20 to 30 days before the puff occurred. The disproportional increase of DNA in these two regions will occur only at the prepupal stage, and the extra amount of DNA is maintained there until the histolysis of the tissue. Swift and Rasch (1962), using histophotometric and autoradiographic methods, showed that disproportional increase of DNA occurs in polytene chromosomes of *S. coprophila;* this also could not be explained only by the asynchrony of synthesis. These results, with some differences in interpretation of data, were confirmed by Gabrusewycz-Garcia (1964) in *S. coprophila.* She believes that with the data obtained by autoradiography the synthesis of "metabolic" DNA is intimately related to the process of replication of the genome (polytenization), but she does not exclude the possibility that the two processes might be independent. The work of Pelc (1962, 1963) suggests that metabolic DNA can be produced in mammalian cells, and also that the process of its production can be independent of the normal cycle of synthesis of DNA which gives rise to a new genome.[5]

In cells of *R. angelae* having polytene chromosomes it is easy to see that chromosomal multiplications or polytenization is a process separated from certain band activities. RNA synthesis, although in this case dependent on the presence of DNA, can occur without a simultaneous DNA multiplication. This can be shown by a comparison of the results obtained with injections of H^3-thymidine in the larva with the results obtained after injection of H^3-cytidine or H^3-uridine. With one injection of H^3-thymidine (about 1.0 μc) in a larva of *R. angelae*, depending on the stage of larval development, from 30 to 100 percent of the cells will show incorporation of the radioactive precursors in its nuclei. As far as we know, similar results are obtained if the larva is killed a few hours or several days after the injection. If DNA turnover exists in these chromosomes, it must be very slow. For the cells of the malpighian tubuli and the intestine, the number of nuclei showing incorporation is smaller, and normally only a few nuclei show incorporation. This is expected because the polytene chromosomes in cells from the malpighian tubuli and intestine are much thinner and normally less active than those from the salivary gland. On the other hand, with one injection of H^3-cytidine or H^3-uridine, the number of nuclei showing incorporation in any one of the above-mentioned times is over 95 percent. These results are obtained even if the larva is killed 30 to 40 minutes after the injection. Since incorporation of H^3-thymidine indicates synthesis of DNA, and incorporation of H^3-cytidine and of H^3-uridine indicates mainly synthesis of RNA, we may conclude from these data that (*a*) DNA and RNA can be synthesized simultaneously in polytene chromosomes of *R. angelae,* and (*b*) synthesis of RNA can occur without the simultaneous synthesis of DNA. As I have discussed elsewhere (Pavan, 1963), a possible explanation of the simultaneous synthesis of DNA and RNA in the same loci of the polytene chromosome is that while some molecules or parts of molecules are producing more DNA, other molecules or parts of them are producing RNA.

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[5] The work of Lima de Faria (1962) shows that "metabolic" DNA is formed in *Tipula oleracea*, and there are good indications that the synthesis of it can be independent of the rest of the genome.

COMPARISON OF THE GROWTH OF TISSUES FORMED BY CELLS WITH POLYTENE CHROMOSOMES WITH TISSUES FORMED BY DIPLOID CELLS

If we compare the growth of a tissue of Diptera having polytene chromosomes, like the salivary gland of *R. angelae,* with the growth of a tissue in which the cells have a diploid number of chromosomes, we find a striking difference. Although the tissues of the former grow by increasing the size of each individual cell (fig. 6, Pavan, 1958), growth in the latter is due to multiplication in the number of cells. Chromosomal multiplication occurs in both tissues, but, whereas in the polytene chromosome cells the newly formed elements stay together with the old ones, in the diploid chromosome cells each chromosomal division is followed by a mitotic process separating the chromosomes and maintaining the diploid number per cell.

It is important to compare the patterns of synthesis in these two types of chromosomes. Synthesis of DNA is rather easy to follow in polytene chromosomes of *R. angelae,* in which the synthesis starts and finishes in several different regions of the chromosomes (Pavan, 1963, and unpublished). This seems to occur in polytene chromosomes of Drosophila (Plaut, 1963; Gay, 1963), but Chironomus may be different (Keyl and Pelling, 1963). Synthesis of DNA is somewhat more difficult to observe in the chromosomes of the diploid cells, because their chromosomes are not individually distinguishable during interphase, when the duplication occurs. Using the autoradiographic method and a short pulse-labeling, however, one can determine what happens during duplication, observing the chromosomes at metaphase when they are very condensed. Here it also appears that DNA synthesis starts and finishes in several different regions of the chromosomes, which suggests that these chromosomes also are formed by several independent units of DNA in regard to the process of duplication.

In the polytene chromosome of Sciaridae, DNA synthesis is not always equivalent in different cycles of synthesis. There are certain phases of larval life during which divisions are more frequent at certain loci than others. For the polytene chromosome of the salivary gland of *R. angelae* which we could observe and follow, the extra synthesis of DNA occurred during or before puff formation, during which much RNA is produced. Our interpretation of this extra synthesis of DNA in some loci of the chromosomes at certain stages of larval development is the following: The activities of the genes during different physiological stages of the cells as well as at different stages of the animal's development must be diverse. There is a time when little or no production of certain genes is needed; other times they are needed in reasonable amounts, and on still other occasions they are needed in great quantities. It looks as if the organisms developed the various ways of controlling these situations by natural selection. The inactivity, or little activity, of certain genes is regulated by the absence of the precursor or by a more general process, manifested by a total or partial condensation of the chromosomal loci. One can easily observe in polytene chromosomes of *R. angelae* that when a band is very condensed, little or no incorporation of radioactive precursors of nucleic acid or protein occurs. On the other hand, great incorporation of all or some of the precursors is always visible when the band is puffed. The mitotic chromosomes at metaphase, when they are very condensed, are metabolically inactive (Pavan, 1964).

For the production of a reasonable amount of RNA, uncoiling of the corresponding chromosome or chromosomal regions, associated with other nuclear and cytoplasmic processes, is enough. For the production of a large amount of certain gene product, one can imagine the development of at least two processes in the polytene chromosomes of *R. angelae*: One must be a system of increasing the speed of the reaction leading to RNA production, the other an increase in the number of templates (genes) responsible for the respective RNA production. This last process can easily be observed at a total or partial genome level in the various cases of polyploidy and polyteny we mentioned. In the polytene chromosome of *R. angelae* the same thing can be seen at the gene level, in the puffs where extra synthesis of DNA takes place.

I do not see any valid reason why this last type of chromosomal behavior cannot occur in cells with a diploid number of chromosomes. The common argument against the comparison of the behavior of tissues having cells with polytene chromosomes with tissues made up by cells with a diploid number of chromosomes is that cells having polytene chromosomes are at the end of their development and differentiation, will not divide anymore, and soon will end their function and disappear. This argument can be eliminated if one remembers that several tissues of mammals (blood-forming cells, epithelial and many glandular cells, and others) have similar behavior. As a matter of fact, approximately 1 percent of our cells are eliminated and rebuilt every 24 hours of our life (Hoffman, 1961). It would be important to know what happens with the genetic material of these cells, and whether the possible changes in its DNA content are the cause or the effect of its differentiation and degradation.

In cells of *R. angelae*, the extra synthesis of DNA in several loci of its polytene chromosomes takes place a long time before the cell shows any sign of degradation.

CONCLUDING REMARKS

The polytene chromosomes of Diptera are excellent material for study of the activities of genes in differentiated cells. The biological characteristics of *R. angelae* permit observations and comparisons of the activities of many gene loci in several tissues and in many different stages of organic development. The gene loci in the polytene chromosomes of Diptera are represented by discs, mainly formed by DNA, which are of different thickness and in different stages of condensation. These discs are distributed in a specific order along the length of the chromosome, and their pattern and distribution are characteristic of each species. We have good data, obtained by the use of radioactive precursors of nucleic acids and of proteins, indicating that when the discs are condensed the corresponding genes are inactive, but when puffed, the respective genes are active. The activities of the bands in one tissue vary with the developmental as well as the physiological stage of the cell and are also different in different tissues. In *R. angelae* and *S. coprophila* it was demonstrated that some gene loci with great activity during a certain stage of organic development show during the process an extra synthesis of DNA not present in the neighboring gene loci. Our interpretation of the particular increase of DNA in these chromosomal loci is that the amount of RNA to be produced at a certain moment is so large that the species developed by natural selection a process to accomplish this great synthesis of gene product, namely, an extra duplication of the gene itself. Processes involving increase of genetic material inside a nucleus, followed by great activity in cell functions, are very common in several species in which polyploidy and polyteny occur. In these cases, the duplication normally occurs over the entire genome, sometimes with the exception of some chromosomes, as in the nurse cells of the ovary of *R. angelae*. In the polytene chromosome, it was possible to demonstrate at the gene level the multiplication of genetic material following or as a consequence of a great cell activity. This shows that during chromosomal duplication there are genes which multiply more than others. It is also probable that gene multiplication can sometimes occur without a corresponding chromosomal duplication. Because of the frequent chromosomal division in polytene chromosomes, it is rather difficult to know whether the multiplication of a special gene can or cannot be (in some cases) separated from the process of polytenization. Although there is no clear case in which this can be demonstrated in Diptera, the work of Pelc (1962, 1963) suggests that this can occur in chromosomes of mammals.

Keyl (1965), measuring the DNA content of homologous bands of polytene chromosomes of two subspecies of Chironomidae, showed that they differ in the quantity of genetic material. Many bands of *Chironomus thummi-thummi* have more DNA than the homologous bands of *Chironomus thummi-piger*: The important point is that the difference in amount of DNA in the band of one subspecies is always 2, 4, 8, or 16 times the corresponding amount in the other subspecies. These results show that the bands in polytene chromosomes are formed by a certain number of units of DNA which are, in relation to the duplication process, independent of the neighboring bands.

In these two subspecies of Chironomidae, measurement of DNA content of the nuclei of spermatocytes differs in content, which Keyl (1965) interprets as representing the difference he finds in the bands of the polytene chromosomes. The work of Levine and van Valen (1962) in polytene chromosomes of *Drosophila pseudoscura* shows that differences in the banding pattern (and probably in DNA content in the bands, if one observes the figures they published) can be obtained as the result of the effect of a recessive gene in homozygosis. The morphology of certain bands and also the banding pattern in a certain region of the polytene chromosome in the individual homozygotes for the gene *salivary* are different from those individuals heterozygous or homozygous for the wild-type gene. If the variations in the polytene chromosomes had correspondent differences in the mitotic chromosomes of the species, it would be expected that the heterozygote would express it, since each polytene chromosome is the product of the multiplication of two homologous chromosomes which paired at early stages of development.

If the gene loci show such an individuality in chromosomes of several species in which this process can be observed, I see no objection to expect similar occurrences in cells of other organisms. It is very important to develop methods which would allow us to know what happens in cells in which there is a great production of one substance or a group of substances, like erythroblasts for hemoglobin, muscle cells for muscle fibers, nerve cells for neurofibrils, and so on. We should know whether the genes mainly responsible for the production of these substances are present in the same number as other individual genes or if they are more numerous. More specifically, the data, available in the polytene chromosomes and in the behavior of many cells to duplicate genetic material when necessary for a large production of cell product, suggest that in these cells, in which a certain group of substances has to be produced in great quantities, the responsible genes could be represented by several copies.

Another important point is that if the duplication of the genes can in some way be independent of the multiplication of other genes, and the number of genetic units produced in each cycle of multiplication of DNA can vary with the developmental stage of the organism, one can think of ways to change the rate of multiplication of specific genes in a chromosome by submitting the cells or tissues to physical, chemical, or biological agents.

My hypothesis—perhaps not easy to accept for the moment—is the result of observations made on the chromosomes of infected cells of *R. angelae* (Diaz and Pavan, 1964, and unpublished). Among the several infections present in cultures of *R. angelae*, two gave results of general importance. One infection is due to a protozoa, probably of the family Microsporidae, and attacks cells of the salivary gland. The infected cells lose control of growth and attain a size 10 to 20 times that of normal cells. The polytene chromosomes of these cells increase proportionally in size, but besides this they show specific changes which in the present case are in the heterochromatic region. These regions are puffed and transformed into a structure looking very much like a nucleolus. The nucleolar organizing region in normal cells is located in the base of chromosome X, but cells of the salivary gland of old larvae never show a typical nucleolar structure. A typical nucleolus, however, can be seen in cells of the salivary gland, the intestine, and the malpighian tubuli of young larvae.

The other infection, probably due to a DNA virus, attacks cells from an accessory intestinal gland; besides an increase in size and in the size of their chromosomes, the infected cells show many polyhedrous crystals that are produced as the result of the infection. The chromosomes of the infected cells, in addition to their enormous size when compared with those of normal cells, show some specific changes in the banding pattern. The most evident changes are related to a kind of gap in certain chromosomal regions, but other changes are also present.

I conclude by saying that chromosomal differentiation exists and was proved in cells of at least two species of Diptera, *R. angelae* and *S. coprophila.* Theoretically there is no valid reason why the same type of differentiation could not occur in some cells of other organisms. The problem is, for theoretical as well as for practical reasons, so important that I thought it worthwhile to discuss it.

RESUMEN

Si se considera el núcleo como un todo, los cambios que es sabido occurren en el material genetico durante el desarrolo son de tres clases principales: eliminación, replicación y manifestación. Los tres tipos de cambios están en ciertas formas representados también al nivel del gen dentro de los cromosomas, como se puede ver en los cromosomas politénicos de Díptera. Los cambios de eliminación se representarían aquí no como eliminacíon física del material genético, sino como inactivación de ciertos genes. Durante determinados períodos de desarrollo algunas bandas se muestran muy condensadas, sin dar señal alguna de actividad, mientras las demás bandas están muy

activas. Este comportamiento de las bandas, que también corresponde a un cambio en manifestación del material genético, es un proceso muy comun. Los cambios de replicación están representados por ciertos *loci* génicos donde la síntesis de DNA es más frecuente que en los *loci* próximos, de manera que la región cromosómica presenta distinta concentración de genes específicos, si se la observa en diferentes etapas de desarrollo larvario o en células de distintos tejidos.

La síntesis extra del material genético en *Rhynchosciara* siempre precede o ocurre durante un período de gran actividad en los *loci* génicos considerados. Esto lo interpretamos como una adaptación del organismo a la necesidad de una considerable cantidad de cierto producto de los genes, produciendo más modelos génicos en el período específico de desarrollo. Se ofrencen argumentos para demostrar que no hay razón valida para que este tipo de cambio no occurra en células diferenciadas de otros organismos. Se debe tener presente esta posibilidad, ya que no hay datos para comprobarla o rechazarla. Como en cada intervalo de 24 horas descartamos y reconstruímos alrededor de un por ciento de las células de nuestro cuerpo, sería de suma importancia saber qué le ocurre al material genético de estas células.

Se presentan argumentos para demostrar que muchas bandas cromosómicas se comportan como unidades independientes en el proceso de duplicacíon, y se sugieren posibilidades de cambiar el patrón de síntesis de genes en los cromosomas mediante el empleo de agentes físicos, químicos o biológicos.

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DISCUSSION

Moses: The more I hear of *Rhynchosciara* and Dr. Pavan's work with it, the more convinced I am that he did not discover the beast. I believe he invented it and had it made to his specifications. This organism is not only a boon to cytologists and geneticists but also a gift to biochemists.

Favelukes: I was not really prepared to talk about this, but since Dr. Pavan has been so kind as to mention it, I would like to submit to the discussion one experiment we have programmed, based on this idea: to test experimentally whether the total complement of DNA and genetic information contained in the fertilized egg cell are transmitted and present as such in all derived cells and tissues, or whether during differentiation some parts of that information are lost. We have thought of doing this by preparing labeled RNA from reticulocytes which are fairly specific for hemoglobin synthesis. This RNA would be hybridized against DNA of either red cells from bone marrow—and in this case, of course, we expect it to hybridize fully—or with the DNA's of other tissues, and this would allow us to find out if in those other tissues the information for hemoglobin synthesis is present. This first idea has been extended, under the suggestion of Dr. Pavan's work, and could be further worked in the following way: If (according to Dr. Pavan's findings with *Rhynchosciara*) there would exist in nucleated red cells an increased amount of the DNA specific for hemoglobin synthesis, then the actual hybridizing capacity of that DNA for hemoglobin messenger RNA would be larger in terms of per milligram of DNA present than if we take another DNA from other tissues. In that hybridization experiment we could test whether DNA's from different tissues have quantitatively different hybridization capacities for the reticulocyte RNA. [Note added in proof: B. J. McCarthy and B. H. Hoyer (Proc Nat Acad Sci USA 52: 915, 1964) have recently shown that the DNA's of different tissues of the mouse have qualitatively similar hybridizing capacities for rapidly labeled RNA's of different organs; they do not put to a test the relative hybridizing of each DNA preparation.]

Edgar: On the mechanism for puffing I proposed the other day, the driving force for condensation was replication and I would like to ask Dr. Pavan if there is any evidence of DNA replication concomitant with the recondensation puffs.

Pavan: Yes, there is DNA synthesis in that area, but I do not know if you can relate these two things, because DNA synthesis seems to start very early in this puff region and for about 4 or 5 days you still have a very high incorporation of tritiated thymidine in the puffing region.

Perry: I would like to ask a question connected with the papers of Dr. Pavan and Dr. Lara: In these advanced stages where the sedimentation pattern of the RNA is shifted from predominantly 45S to one emphasizing the lower S values, do the autoradiographic studies of Dr. Pavan show any differences in the relative incorporation in the micronucleoli versus the chromosome bands? Is there any autoradiographic difference which can be correlated with the shift in sedimentation profile?

Lara: That is what we would like to see, but so far we do not. The shifting patterns were found just a few days ago, even though we have two sets of experiments. Professor Pavan has done autoradiography experiments and has shown some of these here. However, these were made before we learned about the shifting patterns. It will be certainly interesting to find a correlation between the localization of the label in the autoradiography and the shifting patterns.

Perry: Have you ever composed the difference in incorporation between the various nucleosides, for instance, cytidine and guanosine as opposed to uridine and adenosine? In mammalian cells one sees a marked difference between the relative incorporation into ribosomal RNA and messenger RNA, depending on whether one or the other of these pairs of nucleosides is used.

Pavan: This is part of our program for Oak Ridge where we will meet next year.

Reissig: I would like to ask Dr. Pavan whether those zones with greater total activity of tritiated thymidine incorporated also have more specific activity.

Pavan: We have both. Normally when the more conspicuous puffs appear, there is also a great activity in the total nucleus, but the activity in the puffed regions observed by the incorporation of RNA precursors is undoubtedly greater than in the nonpuffed regions of the chromosomes. Does this answer your question?

Reissig: My question is whether the bands where you see much labeled DNA mean greater rate of synthesis in relation to the total amount of DNA present, or is there only more DNA present?

Pavan: Undoubtedly in *Rhynchosciara*, when an extra synthesis of DNA occurs in one specific region of the polytene chromosomes, sooner or later a big puff appears. As far as we know, extra synthesis of DNA in specific regions of these chromosomes precedes or occurs concurrently with the puffing process.

Becker: In answer to the question raised by Dr. Edgar, one should say a little more definitely that, if DNA replication is required for the regression of a puff, then it could certainly not be the explanation for a puff regression or puff appearance and disappearance because some puffs appear and disappear within very short intervals, within which, at a maximum, one replication cycle could take place. So they appear and disappear several times within a matter of a few hours.

Schultz: I have just one point to add to what Dr. Becker has said. In *Drosophila* and *Rhynchosciara*, measurements of DNA synthesis during the puffing cycle have been made by Rudkin. In *Drosophila*, in contradistinction to what he could measure in *Rhynchosciara*, he found no disproportionate synthesis of DNA during the puffing cycle; the specific relationship of a cycle of replication to a cycle of puffing while not completely excluded is at least not plausible at the moment.

Streisinger: Does either Professor Pavan or Dr. Lara have any evidence for the presence or synthesis of ribosomal particles on the chromosome?

Pavan: Not yet. Now going back to Dr. Becker's statements, I think we should be very cautious about the time of synthesis of DNA in polytene chromosomes. Dr. Becker is using some numbers. I would like to have some more data about that because our data are rather different from his. Perhaps in our case it would be about 8 hours for a cycle, although we are not sure about that yet. We would be surprised if it would take several days as Dr. Becker mentions as a possibility for *Chironomus* and *Drosophila.*

The Nucleolus and the Synthesis of Ribosomes [1, 2]

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SUMMARY

In higher organisms genes (cistrons) that direct the synthesis of ribosomal ribonucleic acid (RNA) are located in the discrete regions of the chromosomes associated with the nucleoli. Transcription of these genes results in the formation of large 45S molecules which are subsequently cleaved within the nucleolus to produce 18S and 35S RNA components. The 18S RNA unit combines with protein to form a 40S particle and moves to the cytoplasm in advance of the larger RNA component which remains for a time in the nucleolus and becomes part of a 60S ribonucleoprotein particle. Probably a considerable portion of the nucleolar mass is comprised of such 60S particles. Eventually these particles are also transported to the cytoplasm where they quickly combine with 40S particles to form the 76S ribosomes. At some stage in the genesis of the 60S particle, the 35S RNA precursor is transformed into the 28S RNA component of the ribosome.—Nat Cancer Inst Monogr 18: 325–340, 1965.

RECOGNITION of the fact that the nucleolus represents localized chromosome activity came in the early 1930's when Heitz (*1*) and McClintock (*2*) demonstrated clearly that nucleoli are determined by specific heterochromatic regions of the chromosomes, termed "nucleolus organizers." Some years later when Caspersson and Schultz (*3*) and Brachet (*4*) found that the nucleolus was rich in ribonucleic acid (RNA), the nucleic acid already suspected of being involved in cytoplasmic synthesis, it became natural to suppose that this nuclear organelle played an important role in the expression of genetic information. Indeed, the relationship between the chromosome and nucleolus provided one of the earliest indications that RNA is a primary gene product (*5*).

By 1960, an impressive body of evidence had accumulated which suggested that much of the cytoplasmic RNA was synthesized in the nucleolus. The bulk of the evidence came from autoradiographic studies of the relative rates of incorporation of labeled RNA precursors into the nucleus, nucleolus, and cytoplasm [*see* (*6*) for references]. A consistent finding

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[2] Supported in part by grant 12491 from the National Science Foundation.

was that the nucleolus as well as the extranucleolar portions of the nucleus became labeled earlier than the cytoplasm, and that when the organisms were allowed to continue RNA synthesis in the absence of labeled precursor, the nucleolus lost label while the cytoplasm gained it. The hypothesis was further strengthened by the results of microelectrophoretic measurements (*7*) showing that the base composition of cytoplasmic RNA was nearly identical to that of nucleolar RNA but quite different from that of the RNA in the nucleoplasm.

Rigorous proof of nucleolar involvement in cytoplasmic RNA synthesis was obtained with the demonstration that selective irradiation of nucleoli with an ultraviolet microbeam strongly inhibits the appearance of newly formed RNA in the cytoplasm (*6*, *8*). These irradiation studies, together with quantitative measurements of incorporation kinetics, led to a model that stipulated two systems for RNA synthesis in the rapidly growing cell: a nucleolar synthesis responsible for roughly two thirds of the cytoplasmic RNA and an extranucleolar or chromatin synthesis accounting for the rest (*9*).

SITES OF SYNTHESIS OF VARIOUS TYPES OF RNA

Further elucidation of nucleolar function required that one consider the fact that RNA is not a single molecular species, but rather a mixture of types, each with its own characteristic base composition, size distribution, and degree of lability. Fortunately, suitable methods for the extraction and characterization of RNA had been recently developed so that one was provided with the tools necessary for an attack on this problem. A device of especial value in these studies was the use of critical concentrations of actinomycin D to distinguish between newly synthesized nucleolar and chromatin RNA. Nucleolar RNA synthesis has a much greater sensitivity to actinomycin than chromatin synthesis, so that with a judiciously chosen concentration one can produce cells that manufacture normal amounts of RNA in the chromatin region but which have a greatly reduced capacity to make nucleolar RNA (*10*).

A summary of data comparing rapidly labeled RNA from normal and actinomycin-treated L cells is given in table 1. Under conditions in which the over-all synthesis of RNA was lowered by 45 to 55 percent, the synthesis of nucleolar RNA was reduced more than fivefold. Concomitant with the reduction of nucleolar activity there was a deficiency of the labeled components that sediment at about 45S and 35S.[3] Furthermore, if the treated cells were further incubated in unlabeled medium, there was no measurable labeling of the 28S and 18S ribosomal RNA. Thus, it appears that nucleolar synthesis involves the production of large 45S and 35S molecules which are obligatory precursors of ribosomal RNA (*11*).

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[3] The peaks designated 35S and 28S in this paper were respectively called 38S and 32S in previous publications (*11*, *15*). There is still some uncertainty with regard to the exact "S" numbers. The numbers given here are those obtained in most cases, calculated by proportionality with the 18S peak as a standard.

TABLE 1.—Properties of rapidly synthesized RNA from normal and actinomycin-treated L cells

Source of rapidly labeled RNA*	Relative specific activity	Cellular location (autoradiography)	Sedimentation behavior (sucrose gradient)	Base composition (electrophoresis) Cy/Ad/Gu/Ur	Estimated percentage of messenger RNA† (hybridization)	Fate of label (4-hr chase in presence of actinomycin)
Normal cells	100	⅔ nucleolus, ⅓ chromatin	45S, 35S, and polydisperse	28.1/16.8/31.5/23.5	23–27	28S, 18S (ribosomal)
			4S	28.4/18.6/29.4/23.6		4S (transfer)
Actinomycin-treated cells‡	45–55	¼ nucleolus, ¾ chromatin	Polydisperse	28.5/22.1/27.1/22.3	42–48	4S
			4S	28.7/18.1/28.8/24.4		

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* Cells pulsed for 30 minutes with either H^3-cytidine, C^{14}-cytidine, or P^{32}O$_4$. RNA extracted with phenol and 0.5 percent SDS at 60° C (*11*).

† Messenger RNA used here to connote RNA, not of ribosomal or transfer type, which forms hybrid duplexes with deoxyribonucleic acid (*12*). Hybridization measured by agar-gel technique.

‡ Actinomycin D added to medium at a final concentration of 3.3×10^{-8} M 10 minutes before pulse-labeling

Studies of the kinetics of labeling (*13*, *14*) suggested that 45S RNA is the primary component to be synthesized and that it is subsequently converted to 35S and ribosomal RNA.

Besides a prominent 4S component, the RNA synthesized by actinomycin-treated cells consists of polydisperse components, with a base composition notably different from the ribosomal-like species characteristic of normal pulse-labeled RNA. When allowed to hybridize with excess quantities of deoxyribonucleic acid (DNA), rapidly labeled RNA from actinomycin-treated cells formed almost twice as many duplexes *per unit amount* as did RNA derived from normal cells (*12*). A series of experiments were performed in which increasing amounts of rapidly labeled RNA, prepared from normal and actinomycin-treated cells, were annealed with a constant quantity of DNA. At near saturating levels of RNA the amount of hybrid formed with the two types of preparations was nearly identical, which indicates that the polydisperse components are the only detectable hybridizing species in unfractionated RNA extracts. This strongly suggests that they represent the elements of messenger RNA. From the combined autoradiographic, hybridization, and sedimentation data it was estimated that these components account for 23 to 27 percent of the rapidly labeled RNA in normal cells (*12*).

The 4S RNA synthesized in the presence of actinomycin was shown to have essentially the same base composition as that made in the absence of inhibitor and to exhibit the same kinetics of degradation when hydrolyzed with snake venom diesterase (*15*). Since this 4S RNA is typical of transfer RNA, it was concluded that the chromatin region of the nucleus is also the site of synthesis of transfer RNA.

One explanation for the various RNA species having different sensitivities to actinomycin can be found in a consideration of the sizes of the RNA's being synthesized. If one calculates the maximum number of guanine residues in DNA that could be bound by a particular concentration of actinomycin, assuming that the affinity of the antibiotic for DNA is so great that after a short time of exposure all the actinomycin of the medium will be concentrated by the cells,[4] it follows that 4×10^5 cells per ml exposed to 4×10^{-11} moles of actinomycin per ml will result in a content of 10^{-16} moles of actinomycin per cell. Since there are roughly 10^{-14} moles of DNA guanine per L cell it is seen that, at the very most, only 1 per 100 of the guanine residues could be bound. If the guanines were distributed randomly, the average gap between bound actinomycin molecules would be greater than 400 nucleotides long. It is therefore reasonable to expect that the synthesis of a molecule such as transfer RNA, which has 80 nucleotides, might be quite insensitive to low concentrations of actinomycin. One would also predict that the 45S nucleolar RNA's, being among the largest pieces to be made and having a relatively high proportion of cytosine and guanine residues, should have the greatest sensitivity to the drug. Messenger RNA's of intermediate sizes may or may not be made, depending on the distribution of bound actinomycin molecules. Furthermore, it should be pointed out that, whereas we have, measured the functional integrity of the ribosomal precursor, *i.e.*, its ultimate conversion into the 18 and 28S ribosomal components, we have not measured the functional integrity of the messenger-like components. Some of the polydisperse RNA's made in the presence of actinomycin could well be incomplete fragments of message which, by hybridization criteria, would be indistinguishable from perfect message.

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[4] This is obviously an overestimate, but when less than saturating concentrations are used it closely approximates the situation. For example, with an actinomycin-to-cell ratio 5 times that which is routinely used in our experiments (2 μg/ml actinomycin, 4×10^6 cells/ml), approximately 50 percent of the actinomycin is absorbed from the medium in less than 30 minutes (*16*).

DETERMINATION OF THE GENES RESPONSIBLE FOR RIBOSOMAL RNA SYNTHESIS

Further analysis of the 35S-45S RNA and its relationship to ribosomal RNA was made by using the hybridization technique. Preparations of labeled 35S-45S RNA were purified so as to be relatively free of ribosomal RNA and then allowed to anneal with DNA in the presence or absence of unlabeled ribosomal RNA. The ribosomal RNA competed with 50 to 70 percent of the 35S-45S RNA for sites on the DNA, which indicated that some of the 35S-45S RNA contained base sequences identical to those found in ribosomal RNA (*12*). It was concluded that the conversion from 45S to ribosomal RNA occurs with the preservation of base sequences, and therefore that the transformation process most likely entails a specific disaggregation or a limited chain scission. The 35S-45S RNA's that form hybrid even in the presence of a large excess ribosomal RNA are probably elements of messenger RNA which sediment in this region of the gradient. These could represent large polycistronic messages, or, on the other hand, they might be specific aggregates or complexes of messenger and ribosomal precursor RNA.

The foregoing data strongly suggest that the DNA associated with the nucleolus contains genes which direct the synthesis of ribosomal RNA. That this is true for mammalian cells was shown by McConkey and Hopkins (*17*). In their experiments, 28S ribosomal RNA of high specific activity was allowed to hybridize with DNA extracted from whole nuclei and with DNA extracted from a preparation of isolated nucleoli. The 28S RNA formed 5 times as much hybrid with nucleolar DNA as it did with an equivalent amount of total nuclear DNA, which shows that ribosomal RNA genes are concentrated in nucleolar-associated DNA. Since in these experiments the nucleolar DNA amounted to 3 percent of the total DNA, an increase of over 30-fold should have been observed if all the ribosomal genes are associated with the nucleoli. That only a fivefold increase was found could mean that during isolation of the fractions there was some exchange between nucleolar DNA and non-nucleolar DNA, or that under the conditions of these experiments the nuclear DNA renatured with itself to a greater extent than the total nuclear DNA. But another possiblity is that there are some non-nucleolar genes which code for ribosomal RNA. Since the results discussed in the previous section indicate that the only genes involved in producing ribosomal RNA are those associated with the nucleoli, one would have to suppose that any such non-nucleolar genes, if they exist, do not normally function in producing either nucleoli or ribosomal RNA.[5] From measurements of the fraction of the genome forming hybrid at saturation and a knowledge of the amount of DNA per genome, it has been estimated that mammalian cells contain approximately 2,000 cistrons which code for each of the ribosomal components (*17*, *18*). If only one fifth of these cistrons were active and functioning simultaneously, they could produce 400 molecules of 28S and 400 molecules of 18S RNA. Since there are about one million molecules of each kind per cell, this means that each cistron would have to be transcribed about 2,500 times during one cell generation or about once every 30 seconds. This would allow several milliseconds for the sequential addition of each nucleotide, a reaction rate which is not unreasonable. Hence, 400 cistrons appear to be theoretically capable of supplying a mammalian cell with its total complement of ribosomal RNA.

From the foregoing considerations it is seen that the loss of ability to synthesize ribosomal RNA would involve hundreds to thousands of cistrons. Such an inability was noted in the important experiments of Brown and Gurdon (*20*) who found that in *Xenopus* a single mutation results in a loss of nucleoli and a concomitant failure by embryos to synthesize ribosomal RNA. Such a mutation would therefore have to consist of either a large deletion, a change involving an operator locus, or a deficiency of the factors responsible for the conversion of 45S precursor to ribosomal RNA. Furthermore, there is an obvious implication of a well-regulated control mechanism, since heterozygotes containing only one nucleolus apparently produce normal quantities of ribosomal RNA.

SITE OF THE SPECIFIC DEGRADATION OF 45S RNA

Information about the site of specific degradation of the 45S nucleolar RNA was obtained from a study of cells in which the transformation into ribosomal RNA was retarded due to the incorporation of 8-azaguanine into the RNA molecule (*15*, *21*). In these cells, an azaguanine-containing RNA was synthesized in the nucleolar and chromatin regions at the rate characteristic of normal RNA. Although the RNA exhibited a sedimentation profile indistinguishable from that of normal rapidly labeled RNA, its transformation into the cytoplasmic components was sufficiently delayed so that after a 4-hour chase in nonlabeled medium only about 20 percent of the usual amount of radioactivity was found in the cytoplasm. As can be seen in text-figure 1a, the inhibition occurred almost exclusively in the ribosomal RNA components and principally in the 28S component. Normally the activity of chromatin and nucleolar RNA diminishes during a chase, but in this case there was a tendency for some radioactivity to accumulate in the nucleolus. When the sedimentation characteristics of this RNA were examined, it was found that many of the 45S molecules had clearly been broken down to 35S and 18S components during the chase (text-fig. 1b). These results imply that the 45S RNA is transformed into 35S and 18S components within the nucleolus.

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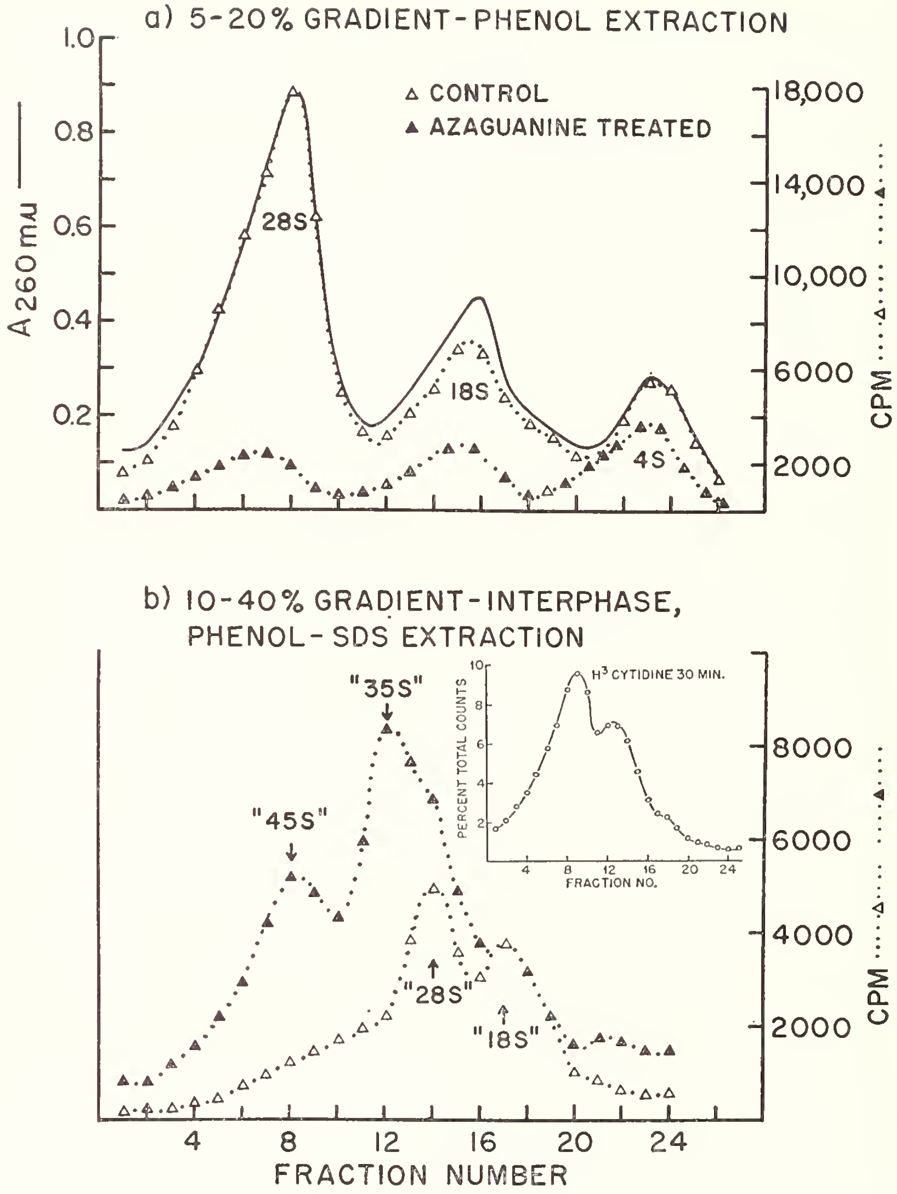
[5] One might even imagine that under certain special conditions these genes could be activated so as to produce nucleoli in other chromosomal regions. An activation of this type was described by McClintock (*2*) and has been recently observed in giant chromosomes obtained from infected *Rynchosciara* (*19*).

After a prolonged chase (~12 hours) the 35S component disappeared and radioactivity appeared in the cytoplasm as 18S and 28S components in an amount equivalent to that expected at 4 hours. Thus it is seen that the transformation of azaguanine-containing RNA into the ribosomal components is not completely blocked, but rather that the over-all process proceeds at a greatly reduced rate, seemingly limited by the step in which 35S RNA is converted to 28S RNA.

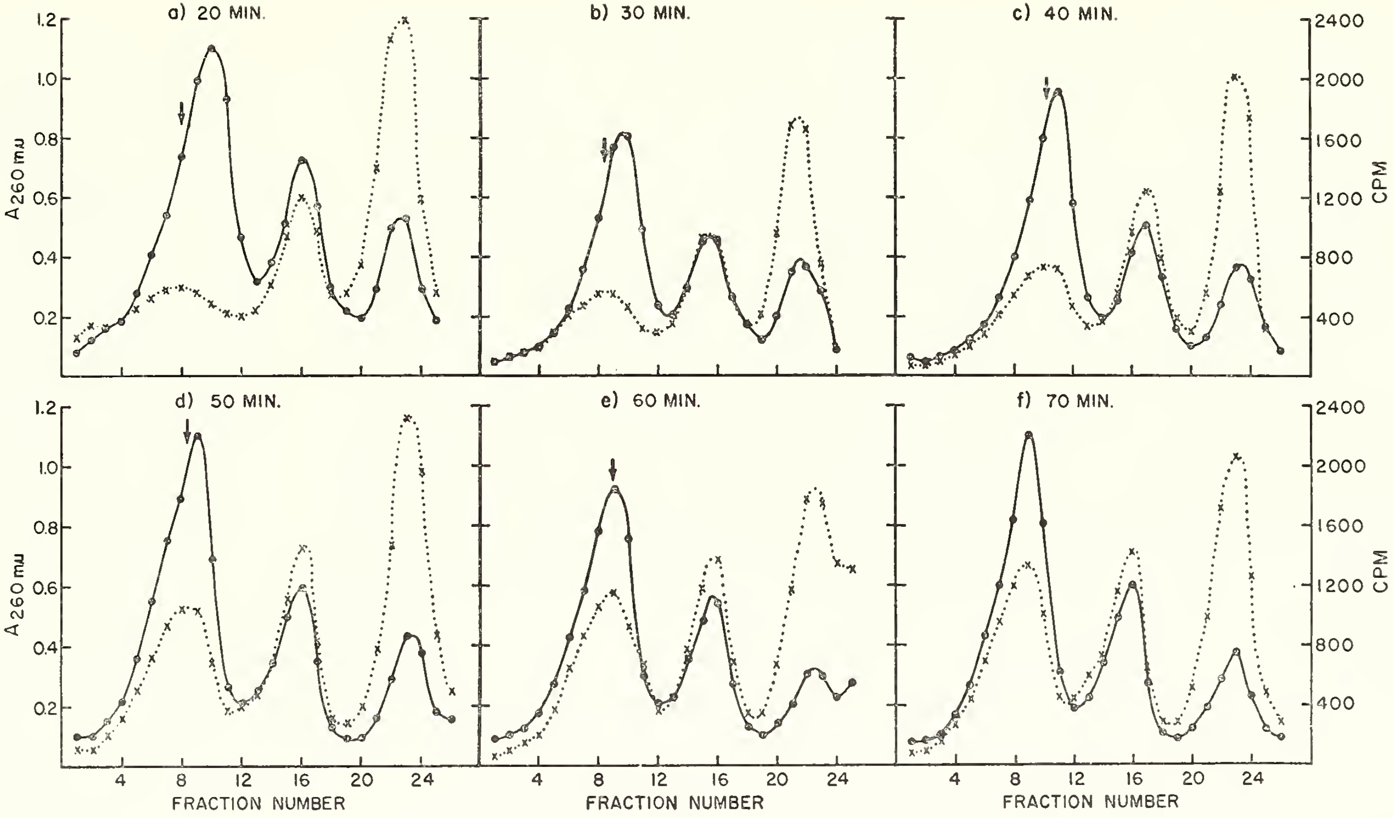
TRANSPORT OF THE RIBOSOMAL COMPONENTS TO THE CYTOPLASM AND THEIR ASSOCIATION WITH PROTEIN

The time course of synthesis of the 28S and 18S RNA components was followed by exposing cells to a brief pulse of labeled precursor and subsequently incubating them for various periods in the presence of unlabeled precursor and actinomycin. The actinomycin was added in order to reduce to a negligible level any further labeling of nucleolar RNA from nondiffusible precursor pools, and, in so doing, to ensure that the observed radioactivity was derived from RNA synthesized during the pulse interval. In text-figure 2 the series of sedimentation profiles show the emergence of radioactivity in the RNA components that are extractable by phenol. Except for 4S RNA, these components are derived almost exclusively from the cytoplasm (*11*, *14*, *22*). Two important facts are evident from these data: 1) Labeled 18S components appear considerably earlier than labeled 28S components, and 2) the earliest radioactive components in the heavier region appear not at 28S but rather at about 35S. As the chase interval is prolonged, there is simultaneously an increase in the size of the radioactive peak and a shift of its maximum from the 35S region into the 28S region. These results indicate that there is a fundamental difference in the pathways by which the two ribosomal components are converted from the form they have immediately following transcription into the form they assume in the cytoplasm.

To investigate the conversion in more detail and to correlate it with the assemblage of whole ribosome particles, an analogous study was made with crude cytoplasmic fractions from cell homogenates. When a post-mitochondrial supernatant fraction was sedimented for an appropriate time on a sucrose gradient, three classes of particles were clearly resolved: a monoribosome peak at approximately 76S, and two smaller peaks at about 60S and 40S (*see* absorbance curves, text-fig. 4). If the supernatant



TEXT-FIGURE 1.—Sedimentation diagrams of RNA from L cells pulse-labeled for 30 minutes with H^3-cytidine and chased for 4 hours with excess unlabeled cytidine. △: Radioactivity in RNA of control cells; ▲: radioactivity in RNA of cells which had 8-azaguanine (0.25 mM final concentration) added to their medium 2½ hours before the pulse. (*a*) RNA extracted by phenol at 5° C; 5 to 20 percent (w/v) sucrose gradient, 3 hours, 39,000 rpm (SW 39). Absorbance at 260 $m\mu$, shown by the *solid curve*, is essentially the same for both preparations. (*b*) RNA extracted from the first interphase of above phenol extraction with 0.5 percent sodium dodecyl sulfate and phenol at 60° C; 10 to 40 percent (w/v) sucrose gradient, 3 hours, 39,000 rpm. Other details of procedure given in (*11*). *Inset:* ○ shows for comparison a typical radioactivity profile of pulse-labeled RNA; the *higher* peak is 45S and the *lower*, 35S.



TEXT-FIGURE 2.—Series of sedimentation diagrams of RNA from cells labeled for 10 minutes with H^3-cytidine and then further incubated for various times with excess unlabeled cytidine in the presence of 3.3×10^{-8} M actinomycin D. Extraction and sedimentation conditions as in text-figure 1a. *Solid lines:* absorbance at 260 mμ; *dotted lines:* radioactivity in counts per minute. *Arrows* designate the position of the heaviest radioactive peak.

fraction came from cells labeled for 30 minutes with H^3-cytidine, the bulk of the radioactivity appeared in the 40S peak and in slowly sedimenting peaks in the 4S region (text-fig. 3a). The 40S component was relatively insensitive to low levels of ribonuclease (text-fig. 3b), and its synthesis was preferentially inhibited with low concentrations of actinomycin (text-fig. 3c). These properties are characteristic of ribosomes or ribosome subunits. Extraction of 40S particles with phenol yielded a labeled RNA sedimenting in a sharp peak at 18S. It may be concluded that the rapidly labeled 40S peak represents a newly formed subunit of the ribosome. Similar results were reported for HeLa cells by Girard *et al.* (*23*).

Supernatant fractions from cells given a 30-minute pulse and then further incubated in the presence of actinomycin exhibited changes in their radioactive sedimentation profiles, such as those shown in text-figure 4. With increasing time there was a progressive decrease in the specific activity of the 40S particles and an increase in the specific activity of the 76S monoribosomes.[6] Although there was a slight increase in the amount of radioactive 60S particles between 30 minutes and 1 hour, at no time was the amount of label comparable to that observed in the 40S particle at 30 minutes, nor did it ever exceed that of the monoribosomes.

Comparing these results with the kinetic data of text-figure 2, one may deduce that the newly formed 18S ribosomal RNA component is rapidly transported to the cytoplasm as part of a 40S ribonucleoprotein particle which eventually combines with a 60S particle to form a monoribosome. During this process there is apparently no change in the 18S component. The delayed appearance of radioactivity in the 28S RNA and the 60S particles of the cytoplasm can most readily be explained by assuming that the nucleolus contains a relatively large pool of 60S particles which acts to retard the flow of radioactive components. At some stage in the genesis of the 60S particles, the 35S RNA is modified so as to sediment at 28S. Two independent results by other workers are consistent with this hypothesis: 1) the finding by Birnstiel *et al.* (*24*) that pea nucleoli contain a relatively large proportion of 60S particles compared to the cytoplasm, and 2) the observation by Girard *et al.* (*23*) that, in contrast to the cytoplasm, rapidly labeled 60S particles are detectable in high yield when extracts are prepared from sonically disruped nuclei.

MODEL FOR THE FUNCTION OF THE NUCLEOLUS

The foregoing evidence may be brought together in the model shown in text-figure 5. The genome, a portion of which is schematically shown by a linear thread, directs the synthesis of various types of RNA. Whereas the majority of cistrons are concerned with the transcription of RNA

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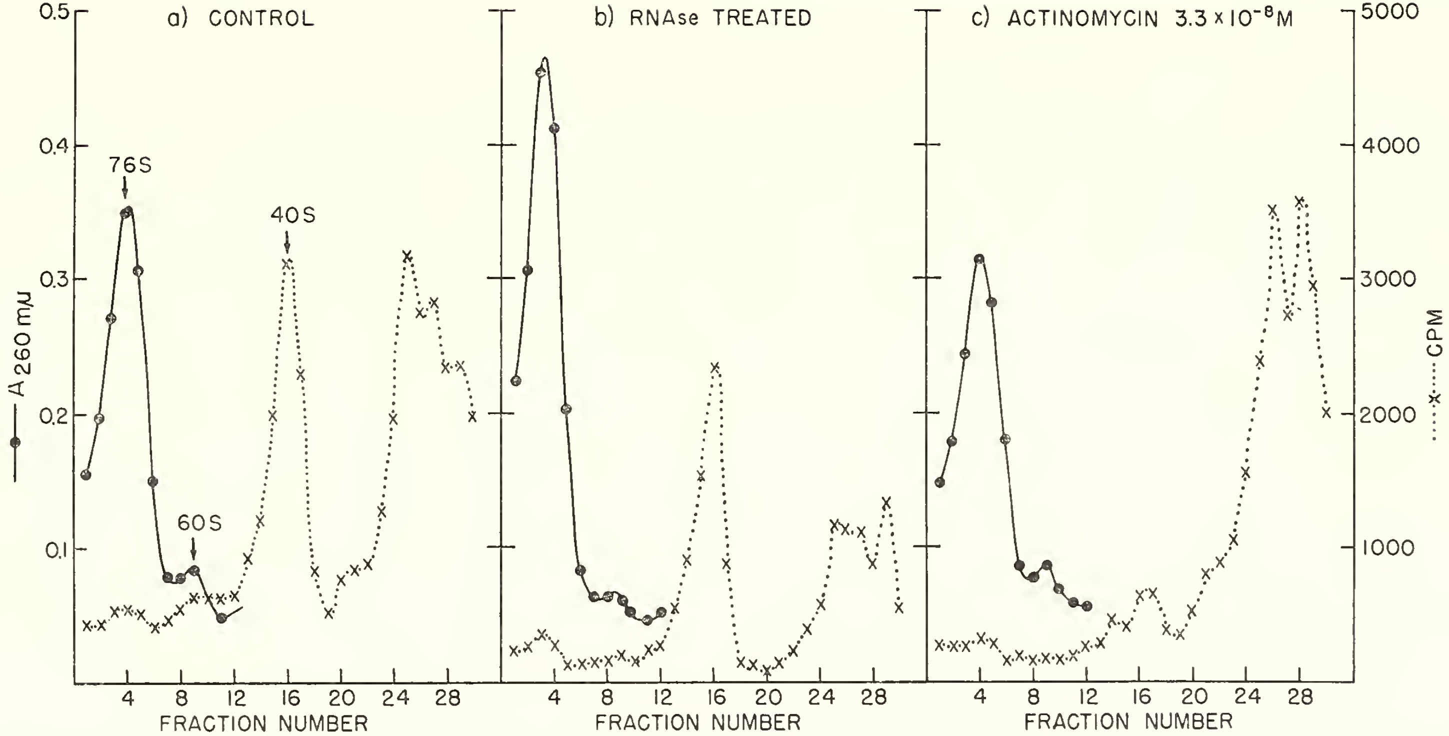
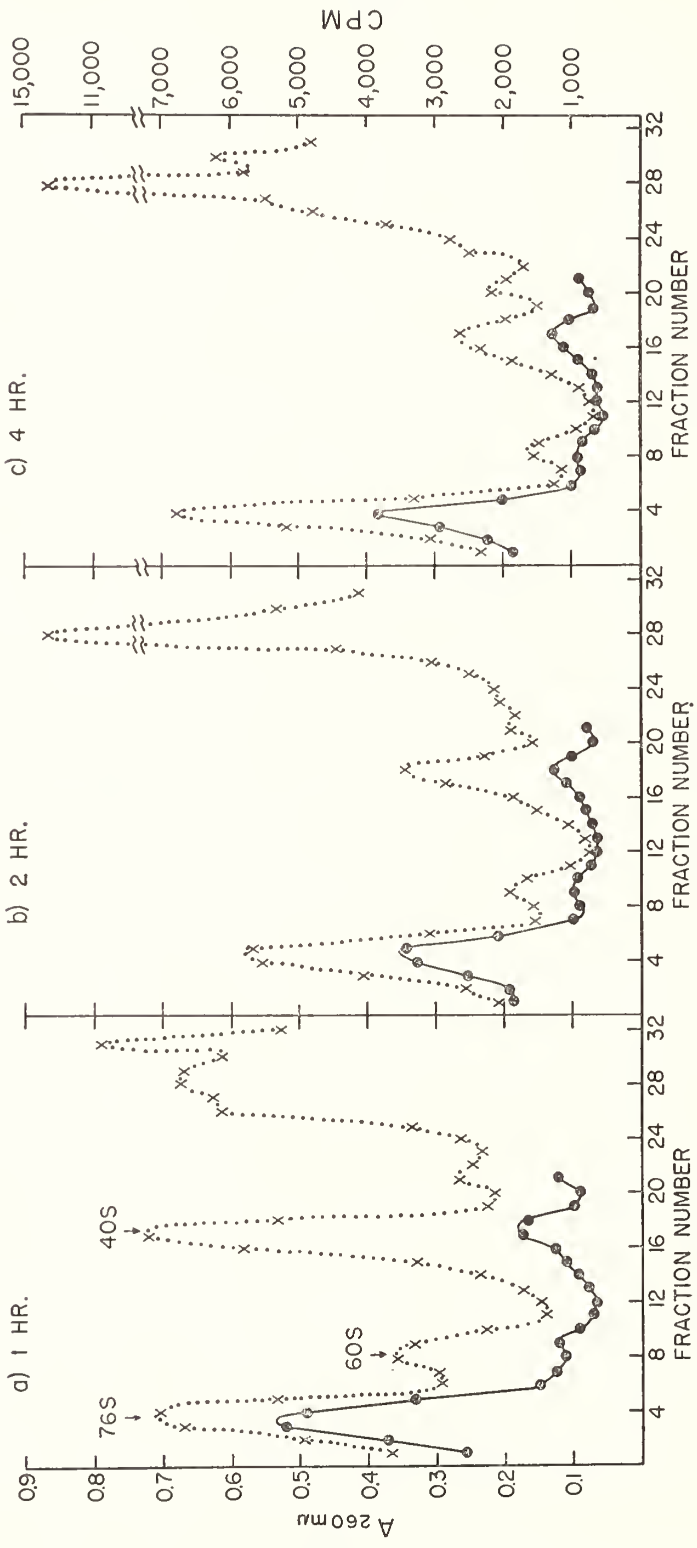
[6] Comparing the counts per minute in each peak on the basis of an equivalent number of absorbance units, we see that between 1 and 4 hours the 40S particles lost about 11,000 counts per minute while the 76S particles gained only about 3,000 counts per minute. This discrepancy can be explained by assuming that some of the labeled 40S particles are incorporated into other units besides the monoribosomes, *e.g.*, into the polyribosomes [*cf* (*23*)], and are not detected under the conditions of our experiment.

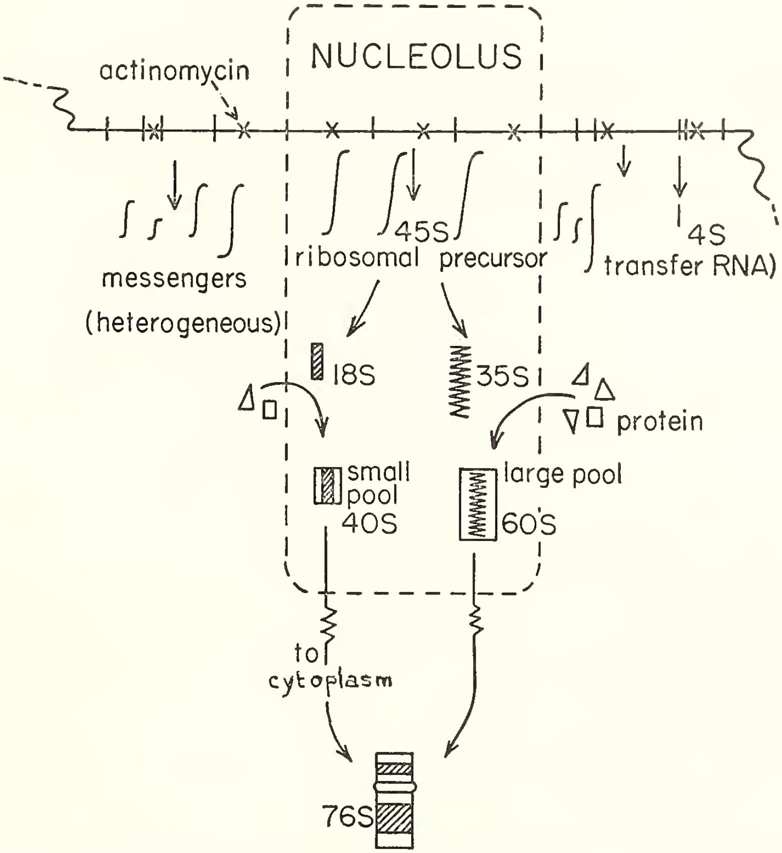


TEXT-FIGURE 3.—Sedimentation diagrams of postmitochondrial supernatant fractions from cells labeled for 30 minutes with H^3-cytidine. About 6 to 10 × 10^7 cells per ml suspended for 10 minutes at 25° C in 0.01 M Tris-HCl buffer, *p*H 7.8, containing 0.002 M $MgCl_2$ and homogenized at 2° C with 15 strokes of a tight-fitting Dounce homogenizer. Following the technique of Spirin and Nemer (personal communication) the homogenate was made 0.15 M with respect to NH_4Cl and 0.25 M with respect to sucrose and centrifuged at 16,000 × *g* for 15 minutes. The supernatant was layered on a 15 to 30 percent (w/w) sucrose gradient containing 0.05 M NH_4Cl and 0.005 M $MgCl_2$ and centrifuged for 12 hours at 25,000 rpm (SW 25). After the absorbance was recorded at 260 mμ, each fraction was precipitated in cold 5 percent trichloroacetic acid. The precipitates were collected on glass-fiber filters and counted with toluene scintillation fluid. Control (*a*) and treated (*b*) are approximately equal portions of the same supernatant; (*a*) was put directly on the sucrose gradient, (*b*) was treated with 10 μg per ml ribonuclease for 1 minute at 25° C before being layered on the gradient. Supernatant (*c*) was prepared from an equivalent quantity of cells which had actinomycin D (3.3 × 10^{-8} M) added to their medium 15 minutes before the pulse-labeling with H^3-cytidine.



TEXT-FIGURE 4.—Sedimentation diagrams of postmitochondrial supernatant fractions from cells labeled for 30 minutes with H^3-cytidine and further incubated for various times with excess unlabeled cytidine in the presence of 3.3×10^{-8} M actinomycin D. Preparation of supernatant as in text-figure 3. *Solid curve:* absorbance at 260 mμ; *dotted curve:* radioactivity in counts per minute.

message, *i.e.*, RNA which is ultimately translatable into specific polypeptide sequences, other cistrons (somewhere between 400 and 2000) are localized in a portion of the genome known as the nucleolar region and are responsible for the production of ribosomal RNA. In addition, there are cistrons located outside the nucleolar region and apparently evenly distributed over the genome, which direct the synthesis of transfer RNA. The synthesis of the various RNA's is differentially sensitive to actinomycin D: ribosomal RNA synthesis being the most sensitive and transfer RNA being the least. One plausible explanation for this differential sensitivity is based on the average size of the gaps between bound actinomycin molecules (*crosses*, text-fig. 5) in relation to the size and base composition of the RNA's being transcribed. The DNA which directs the synthesis of the 45S ribosomal precursor RNA would be one of the largest targets, whereas that which directs the synthesis of the 4S transfer RNA would be among the smallest. However, at this time there is no clear evidence whether an individual 45S molecule consists of multiple units of one kind of RNA or whether it is a complex of different types.



TEXT-FIGURE 5.—Model of RNA synthesis and nucleolar function. For explanation, *see* text.

The nucleolus appears to be the site for the conversion of 45S RNA into 35S and 18S components. The conversion involves a limited degradation in which most of the ribosomal RNA base sequences are preserved. The 18S RNA combines with protein to form a 40S ribonucleoprotein particle while the 35S component becomes part of a 60S particle either before or during its conversion to 28S RNA. The change from 35S to 28S might conceivably be due to a configurational modification of the RNA molecule. It is noteworthy that electron microscope observations of autoradiographs of cells pulsed with H^3-uridine showed that labeled RNA appears first in the fibrous portion and subsequently in the granular region of the nucleolus (*25*). This is entirely consistent with the proposed model. The 40S particles appear to be relatively sparse in the nucleolus, whereas the 60S particles are more abundant. This leads to a differential rate of labeling of the cytoplasmic components, the 40S particles being labeled before the 60S units. When they migrate to the cytoplasm, the subunits quickly unite to form monoribosomes. The previously reported observation (*15*), that 45S RNA can be converted to the 18S and 28S cytoplasmic components in the complete absence of protein synthesis, implies that there is apparently some preformed protein available for complexing with the RNA.

RESUMEN

En los organismos superiores los genes (cistrones) que dirigen la síntesis de ARN ribosómico están localizados en regiones de los cromosomas asociadas a los nucleolos. La transcripción de estos genes resulta en la formación de moléculas grandes de 45S que son subsecuentemente clivadas en sus componentes dentro de los nucleolos para producir los ARN de 18S y 35S. La unidad de ARN 18S se combina con una proteína para formar una partícula de 40S que se dirige al citoplasma. El de 35S permanece en el nucleolo durante un tiempo donde se combina con una proteína dando origen a una partícula de 60S. Es muy probable que una parte muy considerable de la masa nucleolar se componga de partículas de 60S. Eventualmente, estas partículas son también transportadas al citoplasma donde se combinan rápidamente con partículas de 40S para formar los ribosomas de 76S. En algún estadio de la génesis de la partícula 60S el ARN de 35S se transforma en el componente de 28S del ribosoma.

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DISCUSSION

Favelukes: I would like to ask Dr. Perry three questions. The first one is the possibility that in different tissues you might have different ribosomal RNA. I have read a paper by T. Reich *et al.* (in *International Macromolecules*, New York, Academic

Press Inc., 1963) in which they find different compositions of ribosomal RNA's of different tissues of the same organism, while in homologous tissues of different organisms they find a striking similarity in the composition of the respective ribosomal RNA's.

Perry: May I answer that question before you go on to the next one? This may be a true property of the ribosomal RNA itself or it may represent an aggregation between messenger RNA and ribosomal components. It is well known that if you isolate 18S RNA's you have a certain background of messenger components. There may be some nonspecific or specific aggregation between message component and ribosomal RNA component. If this were true, the experiments that you are talking about might represent a contamination, not necessarily an artifactual one, of various RNA messages in various tissues.

Favelukes: If you accept that the messenger RNA associated with a single ribosome represents an amount of perhaps the order of 2 to 3 percent of the total RNA, this would make the explanation rather hard to understand.

Perry: True.

Favelukes: The second question, the 40S and 60S particles that you find or you postulate within the nucleoli are the same 40S and 60S particles that would be obtained by just lowering magnesium concentration of the 76S particles? Would the newly formed 40S particle hybridize with old 60S obtained from magnesium-lowered experiments with ribosomes—could you form hybrid ribosomes?

Perry: I have not done that experiment. It would be a good experiment to do.

Favelukes: The third question is that you mentioned you might need a concomitant protein synthesis to obtain the formation of this 40S particle?

Perry: Not need, I said.

Favelukes: Yes, that you might not need. What is the action of puromycin on this?

Perry: That is the experiment I did. If you add puromycin, you will get transformation of precursor RNA into the ribosomal components.

Favelukes: I will add another question. If you postulate that your 40S and 60S particles have a role in the transport of messenger to cytoplasm, would you tend to believe that perhaps it is only the 40S particles that have that role, because in the experiments with nucleoli you find that the messenger RNA is associated with the 40S particles?

Perry: I tried to demonstrate this. I did an experiment in which, instead of looking at the smaller particles, we analyzed the polysome region. And so one might now ask, what is the order of appearance of RNA in the polysome region? As you predict, it has been reported that 18S, presumably as part of the 40S component, appears on the polysome first. Although I have tried this experiment several times, I cannot repeat this result. I get either a concomitant appearance or the 28S appears first. We do not know what the nature of this discrepancy is, so I think we have to regard it as an open question.*

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*Note added in proof: Further experiments showed that our failure to observe early labeling of 18S RNA in the polysomes was because we used phenol, rather than SDS-phenol to extract the RNA. For some reason, the 28S RNA is more readily extractable under these conditions than is the 18S RNA.

Symposium on Structure and Function of Genes and Chromosomes: A Summation[1, 2]

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ANY ATTEMPT to summarize in its entirety a Symposium as comprehensive as the present one constitutes a formidable and, in some respects, an almost impossible task. Consequently, when invited to undertake this responsibility, I indicated that I would accept provided I could interpret my task in a somewhat restricted fashion. Therefore, I shall not attempt to discuss critically and in detail each of the papers presented at this meeting. Rather I shall emphasize what I consider to be a central theme of these discussions—namely, the evidence for basic similarities (or differences where they exist) in the organization and functioning of the genetic material in the two principal groups of organisms. In addition, I shall take this opportunity to discuss some recent results of studies by my colleagues and me on the functional organization of certain genetic systems in Neurospora—especially as these results relate to what I consider to be the central theme which I have indicated.

The participants in this Symposium have been considering problems of the structure and function of genes and chromosomes at all levels of evolutionary complexity. In general it seems clear from the remarkable progress which has been made in genetics and cytology during the past decade or so that this procedure is, in fact, justified. Certainly the overwhelming mass of evidence supports the view that a substantial unity exists among all organisms with respect both to the gross aspects of their cellular organization and to the composition and mode of action of their genetic materials.

However, at the same time it is apparent that important differences do exist. Unquestionably the clearest discontinuity evident in organismal evolution is that separating the *procaryotes* and *eucaryotes*. The evidence is clear that fundamental differences at the level of ultrastructure distinguish these two groups of organisms (*1*). Of particular significance for this Symposium are the differences so far indicated in organization, mechanisms of transmission, and, to some extent, of function, which dis-

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[1] Presented at the Symposium on Genes and Chromosomes—Structure and Function, Buenos Aires, Argentina, November 30 to December 4, 1964.

[2] The original research reported in this paper was supported, in part, under contract AT(30–1)3098 with the U.S. Atomic Energy Commission.

tinguish the chromosomes of viruses and bacteria from those of eucaryotes. For example, differences in transmission mechanisms are clearly indicated by the research on the colicins described in the paper by Nagel de Zwaig and Antón.

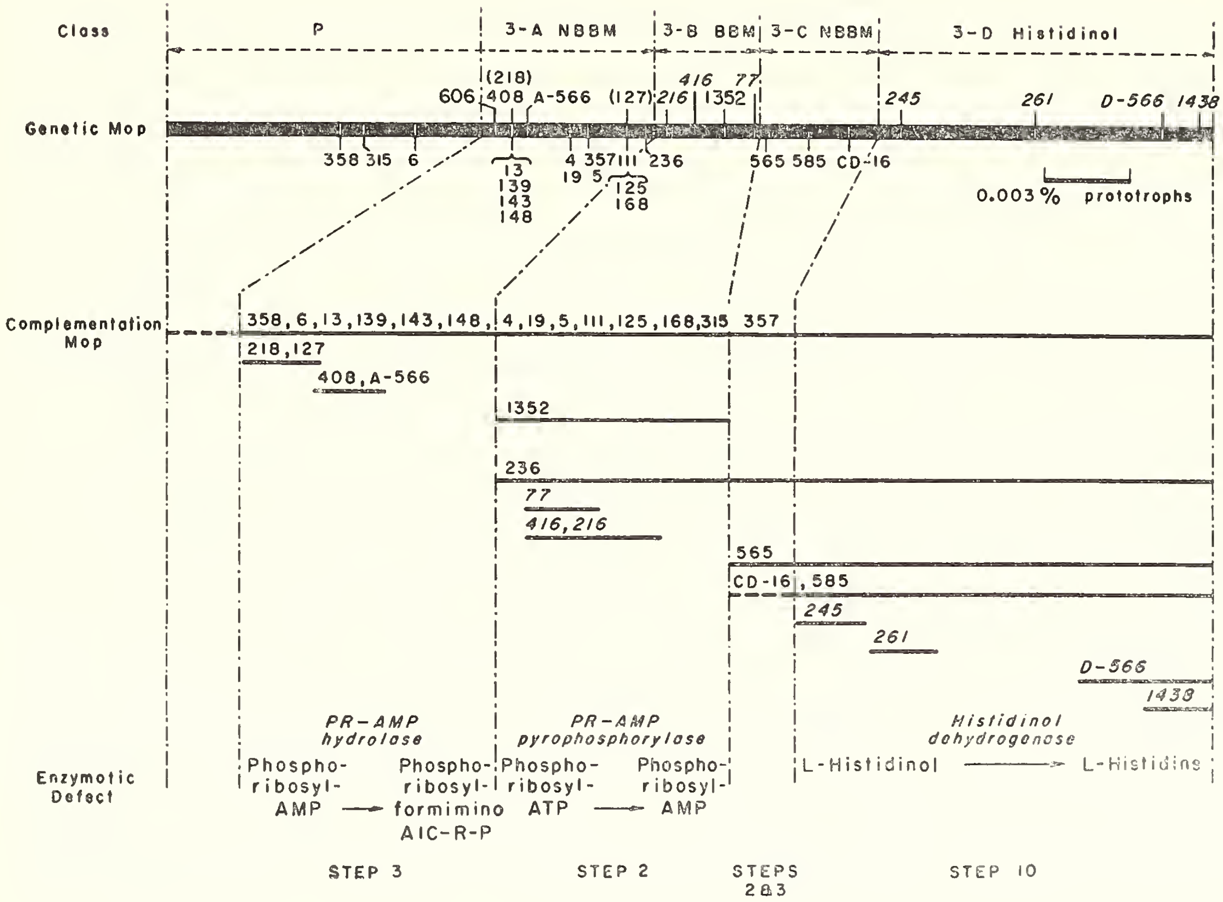
OPERONS IN PROCARYOTES AND EUCARYOTES

Initially I would like to consider in particular the remarkable evidence, first obtained by Demerec in Salmonella, that suggests differences in the functional organization of bacterial chromosomes as compared with those of higher plants and animals. I am referring to the now well-known clustering in numerous instances of genes with related functions, *i.e.*, those controlling different steps in the biosynthesis of particular cell metabolites. As indicated in his paper, Demerec has obtained evidence from studies of a large sample of auxotrophic mutants that approximately 75 percent of the gene loci in *Salmonella typhimurium* are arranged in clusters of two or more in the bacterial chromosome.

This clustering of genes has become generally interpretable in functional terms on the basis of a type of supragenic organization most clearly expressed in the "operon" concept of Jacob and Monod (*2*). Several examples of operons are now known in bacteria. One of the distinctive functional differences distinguishing procaryotes and eucaryotes has in fact resided in the presence, in at least certain bacteria, of clusters of genes functioning as operons and the apparent absence of such functional units in eucaryotes.

It now appears, however, that such clusters of genes do occur in eucaryotes and that they are, at least in certain instances, organized into systems functionally similar, if not identical, to the operons of bacteria. I would like to describe briefly the evidence obtained within the last 2 years for the existence of such systems in *Neurospora crassa*. The best established case is that involving three genes controlling histidine biosynthesis. Our early interest in histidine mutants in Neurospora was renewed as a result of the studies of Ames and Hartman and their colleagues (*3*, *4*) on the histidine operon in Salmonella, which includes all the structural genes involved in histidine biosynthesis in that organism. In connection with these studies, Smith and Ames (*5*) provided new evidence concerning the early steps in the histidine biosynthetic pathway. Thus it became possible to determine the biochemical relationships of all the histidine mutants in Neurospora. On the basis of these studies, evidence was provided by Ahmed (*6*) that mutants in the *histidine-3* (*hist-3*) region are concerned with three enzymes controlling three distinct reactions in histidine biosynthesis—steps 2, 3, and 10.

Combined complementation, recombination, and biochemical studies (*7–9*) have established the existence in the *hist-3* region of three (or possibly 4) discrete groups of mutants arranged in colinear sequences on complementation and genetic maps of the *hist-3* region (text-fig. 1). These



TEXT-FIGURE 1.—The *histidine-3* (*hist-3*) operon in *Neurospora crassa.* The correlation of the genetic map with the complementation map and with enzymatic defects is indicated. The *numerals above the genetic map* refer to complementing mutants, whereas *those below* designate completely or partially noncomplementing mutants (referred to as pleiotropic or polarity mutants); *numerals in parentheses* (*or brackets*) refer to mutants whose locations with respect to closely adjacent mutants on the genetic map are not yet precisely established. For further details, *see* text and (7). [This text-figure incorporates certain corrections of data originally reported in (7).]

3 groups apparently correspond to three different structural genes specifying the polypeptides controlling the third, second, and terminal (histidinol dehydrogenase-catalyzed) steps. In addition to the localized complementing mutants, other types of mutants occur. These are completely noncomplementing mutants, which indicate the pleiotropic loss of all three activities, or partially complementing types exhibiting a polarized, pleiotropic loss of progressively more distal functions—to the right on the complementation map. Genetic data indicate a localization of the completely noncomplementing mutants at the left end of the genetic map either proximal to or among the mutants in the first structural gene.

The biochemical evidence for functional heterogeneity, together with the genetic and complementation evidence for integrated and polarized activity, lead to the conclusion that the *hist-3* region in Neurospora constitutes an operon, *i.e.*, a polarized unit of coordinated transcription as defined by Jacob and Monod (*2*). Present results suggest that the expression of this entire region is initiated by an element—the promoter (P)—localized at the extreme left. The genetic information encoded in the sequence of bases within the entire *hist-3* region is presumably transcribed into a single polycistronic messenger RNA molecule (m-RNA). Translation of this m-RNA is also believed to proceed sequentially from left to right, specifying the amino acid sequences of three polypeptide chains. (Conclusive evidence for the physical separation of the three different activities as distinct proteins has not yet been obtained in Neurospora.)

These observations on the *hist-3* group of mutants in Neurospora suggested that it might be of interest to examine the relationships of histidine mutants in other fungi. Studies on yeast (*Saccharomyces cereviseae*) by Fink (*10*) have in fact indicated that a situation remarkably similar to that in Neurospora exists in yeast. Although, as in Neurospora, the majority of loci controlling histidine biosynthesis are scattered about the yeast genome, the same three enzymatic steps (2, 3, and 10) are controlled by a cluster of three genes. Complementation, recombination, and biochemical studies (*11*) indicate that this cluster has essentially all the characteristics described for the *hist-3* operon in Neurospora. Indeed, the two operons essentially appear to be completely homologous on the basis of presently available genetic and biochemical data.

The detection of a histidine operon in both Neurospora and yeast encouraged us to investigate other situations that might represent operons. The most obvious candidate in Neurospora was the cluster of genes first studied by Gross and Fein (*12*), in which mutations produced polyaromatic auxotrophs requiring tyrosine, phenylalanine, tryptophan, and paraaminobenzoic acid. Complementation, biochemical, and preliminary recombination studies have been performed with over 100 new polyaromatic auxotrophs. On the basis of present evidence, one group of these mutants constitutes an operon basically similar to, but more extensive than, the *hist-3* operon.

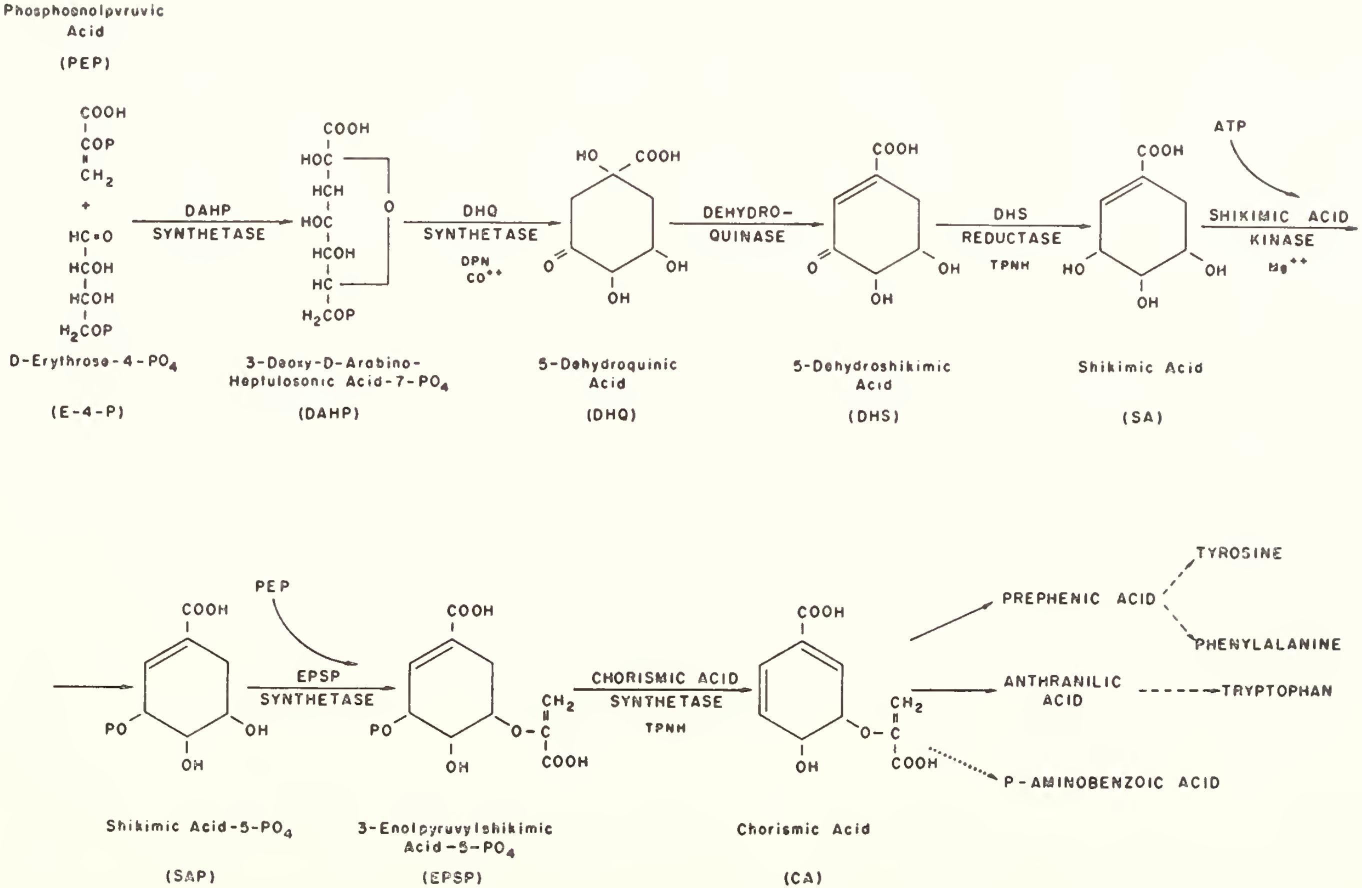
The organization of this aromatic (*arom*) operon, on the basis of present evidence from complementation and biochemical studies, is described in text-figures 2 and 3. Text-figure 2 indicates the biosynthetic reactions prior to chorismic acid in this pathway (*13*). Text-figure 3 indicates the complementation map of the *arom* operon in relation to the five reactions (2 through 6) controlled by the five structural genes within the operon.

The complementation studies with these *arom* mutants provide direct evidence for the existence of four (and presumptive evidence for five) distinct groups of mutants, presumably the result of missense mutations in separate structural genes coding for five different polypeptides (enzymes). In addition, several different categories of polarity mutants have been detected, including a group of completely noncomplementing mutants equivalent to the single *arom-2 Ab* mutant (C 161) of Gross and Fein (*12*). The preliminary ordering of the individual structural genes within the operon (text-fig. 3) is based on complementation evidence from partially complementing polarity mutants and on biochemical evidence from assays for dehydroquinase activity in polarity mutants. In particular, the placement of the presumptive structural gene coding for dehydroquinase (for which no simple missense mutations have yet been obtained in Neurospora) is based on the absence of activity for this enzyme in certain noncomplementing polarity mutants and its presence in other noncomplementing and partially complementing polarity types.

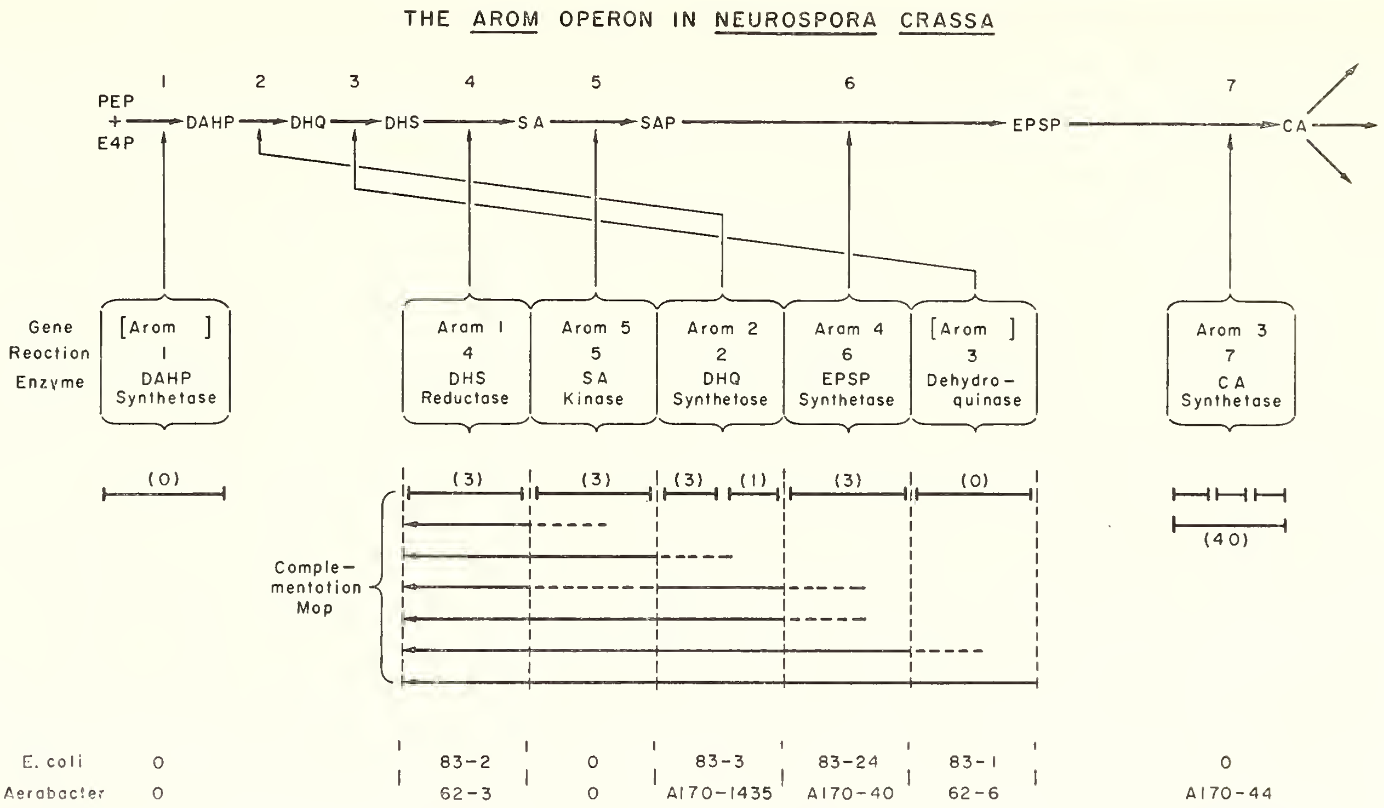
The identification of particular structural genes in the operon as coding for specific enzymes is based on biochemical studies involving either direct enzymatic assays or complementation tests *in vitro*, in which Neurospora mutant extracts have been combined with extracts of bacterial mutants known to be defective for particular reactions (indicated in the lower part of text-fig. 3) (*14*). The biochemical evidence also indicates that the polarity mutants of the completely noncomplementing category have lost all five activities for the enzymes controlling reactions 2 through 6.

Present genetic evidence does not yet permit an ordering of the various structural gene mutants or of the polarity mutants on the recombination map of the operon. However, it is clear that all mutants, including the two newly discovered groups of structural gene mutants deficient for particular enzymatic activities, *i.e.*, for shikimic acid kinase and for dehydroquinic acid synthetase, are all very closely linked, approximately four crossover units to the right of the peach marker in the right arm of linkage group II.

In summary, presently available complementation, biochemical, and recombination data support the conclusion that the cluster of polyaromatic auxotrophic mutants constitutes a second operon in Neurospora comparable, in its general organization, to the *hist-3* operon. Presumably the information encoded in the DNA segment constituting this operon is transcribed in a polarized fashion via a single polycistronic m-RNA which, in turn, is translated by the polyribosome complex, again in a polarized fashion, into five distinct polypeptides. Present evidence suggests that transcription (and subsequent translation) is initiated at the right end of the operon (text-fig. 3), adjacent to the presumptive structural



TEXT-FIGURE 2.—Reactions in the biosynthesis of chorismic acid (in the polyaromatic pathway) in *Neurospora crassa*.



TEXT-FIGURE 3.—The aromatic (*arom*) operon in *Neurospora crassa*. The present preliminary complementation map and the correlation between this map and enzymatic defects in the biosynthetic pathway are indicated at *top* of the figure. *Dotted lines* indicate restricted complementation reactions. The designation of the gene coding for dehydroquinase (DHQ) synthetase as *arom 2* follows the recommendation made by Gross and Fein (*12*). *Numerals in parentheses* are the approximate number of mutants of a particular type. Enzymatic defects in mutants of *Escherichia coli* and *Aerobacter aerogenes* used in complementation tests *in vitro* with Neurospora mutants are indicated in the *lower portion*. The presumptive gene(s) for the first reaction and the identified (*arom 3*) gene for the seventh reaction, neither of which is within the *arom* operon, are included. For further details, *see* text.

gene coding for dehydroquinase, and proceeds sequentially from right to left. On this basis, the class of completely noncomplementing mutants (equivalent to operator negative types) should be localized on the genetic map of the operon either distal to (to the right) or within the limits of the dehydroquinase structural gene. Additional mutational and recombination studies presently underway should serve to test this hypothesis.

The evolutionary relationships of these two operons in Neurospora to the presumably more primitive analogous systems in bacteria constitute intriguing problems (*15*). It is tempting to consider that the histidine operon in Neurospora (and also in yeast) represents a "vestigial" portion of the more extensive histidine operon characteristic of certain bacteria. If this is indeed true, several genetic rearrangements must have occurred during evolution in order to bring together, in their present relationships in the operons of the two species of fungi, the three structural genes presumably homologous with those genes coding for comparable enzymes in Salmonella.

The evolutionary relationship of the aromatic operon in Neurospora to comparable systems in bacteria is still quite obscure. Present incomplete evidence suggests that most of the structural genes controlling the five enzymes comparable to those within the *arom* operon in Neurospora are not closely linked in *E. coli* (*16*), although some may be closely linked in *Bacillus subtilis* (*17*). Hence the *arom* operon in Neurospora may represent either the retention of an original association no longer present in most bacteria, or possibly a new functional association of these genes that became established during the evolution of the fungi, presumably on the basis of some novel, but still unclarified, selective advantage.

IMPLICATIONS OF OPERON SYSTEMS FOR THE ANALYSIS OF GENETIC UNITS OF FUNCTION BY COMPLEMENTATION

The results of complementation and recombination studies with operon systems in Neurospora and bacteria have interesting implications for the use of complementation analysis in defining a genetic unit of function as well as for the mechanisms involved in complementation, especially allelic complementation. Some of these problems are indicated in the papers presented by de Serres, Reissig, and Edgar. As has become quite evident in the past few years, the presence of allelic complementation can lead to difficulties in the application of the cis-trans test in defining the cistron as a basic unit of genetic function. In some systems, it is clear that the phenotypes of certain combinations of allelic mutants may be indistinguishable from the wild type, at least with respect to the usual levels of phenotype available for investigation. When, however, such studies can be carried out at the enzyme level, quantitative or qualitative differences are regularly detected. For example, as indicated in the paper by Reissig, the aspartic transcarbamylase enzyme produced in heterocaryons between certain complementing alleles of the *pyr 3 N* structural gene is much more thermolabile than is the same enzyme produced by the wild type.

Even in the absence of biochemical evidence, if large numbers of mutants (of initially indistinguishable phenotypes) are available for study, it is typically found that these mutants group themselves into a limited number of arrays between which complementation regularly occurs for all mutant combinations, whereas within each array—although certain pairs may exhibit complementation—there are also numerous mutants which are completely noncomplementing in all combinations. This pattern is characteristic of the conditional lethal mutants studied in bacteriophage T4 by Edgar. In such situations the noncomplementing types (*e.g.*, amber mutants in T4) serve to unify the individual groups of mutants into single functional groups—cistrons or genes—defined as a "segment of a genome which contains the hereditary information determining the primary structure of a polypeptide chain." The failure of complementation between certain mutants in two distinct genes, as discussed by de Serres for the purple adenine 3A and 3B mutants in Neurospora, does not constitute an exception to the expected complementation pattern, since the evidence from his studies indicates that such noncomplementing, nonallelic mutants possess overlapping areas of genetic damage.

The detection of genetic systems functionally integrated on a supragenic level, as in the case of operons, introduces certain new complexities into an analysis of function by the cis-trans complementation test. The presence in such systems of a group of noncomplementing mutants may initially appear to indicate that such a system consists of a group of allelic mutants in a single gene. However, sufficient complementation and recombination evidence, even in situations where direct biochemical evidence is lacking, should serve to distinguish an operon from a single structural gene characterized by allelic complementation. The three major distinguishing features are as follows: An operon consists of a cluster of two or more groups of mutants, between which the type of complementation observed is characteristic of mutants in different genes, but within each of which the mutants exhibit behavior characteristic of allelic complementation; complementation data provide evidence for various types of polarity mutants reflecting the occurrence of polarized translation of a polycistronic message; complementation and recombination data establish the existence of a special class of polarity mutants that are completely noncomplementing and which are clustered near one end of the genetic map of the region. These features are in marked contrast with the situation for a single structural gene coding for a single polypeptide, which is characterized in general by a nonpolarized distribution of mutants on the complementation map, by a generalized scattering on the genetic map of the mutational sites representing noncomplementing mutants, and by at least some pairs of complementing mutants (where allelic complementation occurs) that form heterocaryons with phenotypes not equivalent to the wild type (*18*).

GENES AND CHROMOSOMES

FURTHER BASIC SIMILARITIES BETWEEN GENETIC SYSTEMS IN PROCARYOTES AND EUCARYOTES

The results previously discussed provide new evidence for basic similarities in the functional organization of the genetic material in procaryotes and eucaryotes. Other basic similarities are indicated in the paper by Streisinger. For example, it now seems highly probable that the genetic code is a universal triplet code characterized by considerable degeneracy. In addition, there is increasing evidence that the one gene-one enzyme (or one cistron-one polypeptide) hypothesis and the sequence hypothesis are universally applicable. The one cistron-one polypeptide hypothesis is strongly supported by the studies reported in the paper by Schwartz dealing with esterase mutants in maize. His evidence that isozymes characteristic of an allelic series arise as a result of charge differences serves to clarify this and similar situations which initially appeared to be in potential conflict with this hypothesis in its simplest form.

The discussion by Streisinger also indicated that there is increasingly convincing evidence for the occurrence in all organisms of identical categories of mutational change at the molecular level. The evidence for missense, as well as for nonsense mutations, is quite good. Streisinger's own evidence with the lysozyme system for mutations presumably resulting in reading frame shifts leading to changes in several amino acids is of particular interest. However, as he indicated, there remain a number of problems relating to the control of reading, both in transcription and in translation. For instance, the nature of the so-called modulating mutations postulated by Ames and Hartman (*3*), which appear to interfere with translation but do not necessarily stop it completely, remains to be clarified. In addition, the manner in which reading is initiated and terminated is not clear, nor is it yet evident, for example, whether the transcription of a polycistronic message is "in register" throughout.

CHROMOSOMAL ORGANIZATION AND BEHAVIOR IN EUCARYOTES

So far in this discussion, emphasis has been given to the similarities in the organization and action of the genetic material of all organisms, both procaryotes and eucaryotes. However, these basic and very important similarities should not cause us to overlook the obvious differences that exist. It is perfectly clear that the chromosomes of eucaryotes differ in many important respects, structurally and chemically, from their counterparts in procaryotes. Even in the latter group there is ever-increasing evidence, as discussed by Edgar, for a greater degree of structural (and possibly chemical) complexity than was originally thought. Indeed, the "chromosomes" of bacteriophages and bacteria appear to be more than "just a DNA molecule." They may be either linear or circular and can have numerous special features, such as redundancy, sticky ends, swivels, etc.

Unquestionably, an even greater complexity exists with respect to the organization of chromosomes in eucaryotes. However, it is clear from the papers by Miller, Peacock, Sotelo, and Wolff, and from the resulting discussion, that differences of opinion exist with respect to the degree of this complexity. Despite the clear differences of opinion it also seems evident that certain basic areas of agreement exist. All participants appear to agree that the smallest unit in a chromosome is a DNA double helix and that replication is semiconservative.

The major differences of opinion center about the number of strands, here defined as the number of DNA double helices per chromatid, and the nature of the molecules responsible for chromosome continuity. The controversy relative to the number of strands is an old one. For example, consider the various points of view presented at the Cold Spring Harbor Symposium on "Genes and Chromosomes: Structure and Organization" in 1941 (*19*). These various viewpoints could be characterized in those preWatson-Crick days in terms of three major hypotheses: the "conservative," "semiconservative," and "radical," which held, respectively, that an anaphase chromosome consisted of 1, 2, or 4 (or even more) strands. In the past few years many cytologists appear to have adopted the conservative view, but within the past year or so some of these former conservatives seem to have adopted a semiconservative, if not radical, position.

I shall not attempt to review all the arguments for the two major opposing views currently receiving most support. Evidence for a single DNA double helix per chromatid is presented by Miller, whereas principal support for the presence of two DNA double helices per chromatid is provided in the papers by Peacock and Wolff. Although it may seem inappropriate for a summarizer to take sides, even mildly, in such a situation, I will express the opinion that the weight of present evidence supports the hypothesis that the chromosomes of eucaryotes consist of a single DNA double helix per chromatid. I feel that the lampbrush chromosomes of amphibia are presently the most suitable material for obtaining information relative to the "primary" and indeed "quaternary" structure of chromosomes—in the useful terminology suggested by Streisinger. I do not wish to imply that I feel the issue is closed, however. Clearly more evidence will be required. For instance, it would be most useful to have studies involving both enzyme digestion and thymidine labeling (relative to replication) performed on the somatic chromosomes of salamanders.

Perhaps somewhat less controversy centers about the problem of chromosome continuity. There is much evidence suggesting that a series of tandemly arrayed DNA molecules, rather than one continous DNA double helix per chromosome, is present (*20*). However, it is still not clear how the physical continuity of individual chromosomes can be maintained by linking molecules other than DNA, such as protein, which are incapable of replication.

In addition to the unsolved problems of chromosome structure, it was evident from the papers by Grell and Sandler that additional problems remain relative to chromosome behavior at meiosis. The results discussed by Grell suggest the need for a re-evaluation of the entire sequence of events at meiosis, especially as they relate to the interrelationships of chromosome replication, synapsis, crossing-over, and segregation. Sandler described the fascinating features of ring chromosome behavior in Drosophila and suggested that the apparent stability of rings results from the occurrence of exchanges during ring synapsis at meiosis.

Another aspect of chromosome behavior was described in the paper by de Lozzio and Valencia. Their extensive studies with ascites tumor cells indicate that the relationships between X-ray-induced mitotic delay, cell death, and chromosome aberrations are clearly complex. Agents known to inhibit cellular respiration, DNA synthesis, or protein synthesis appear to act at different times in the cell cycle in relation to the three types of radiation-induced cellular damage studied.

FUNCTIONAL ORGANIZATION OF CHROMOSOMES IN EUCARYOTES

The last major topic of the Symposium dealt with the functioning of chromosomes in eucaryotes. This is, of course, one of the major frontiers of modern genetics, especially in relation to problems of development and differentiation. Lindsley discussed the chromosome as a functional unit, reviewing the evidence for the coordination of function for entire chromosome sets, for entire individual chromosomes (*e.g.*, the X chromosomes in female mammals), and for chromosomal segments (*e.g.*, as manifested in various types of position effects). Activity versus inactivity is definitely associated cytologically with extension versus compaction of chromosomes, although the nature of the control mechanisms is still unclear.

Detailed evidence relating to the functioning of specific regions of chromosomes was discussed in papers by Becker, Pavan, and Perry. Of major interest is the possibility of associating specific units of function with specific morphological units in particular chromosomes. This approach may lead to a restatement of the one-gene-one-enzyme hypothesis as the "one-gene-one-band" or the "one-gene-one-puff" hypothesis. However, present evidence does not yet establish such a direct one-to-one relationship. In fact, it seems more probable that the morphological segments constituting chromosome bands in Dipteran salivary gland chromosomes, or loops in amphibian oocyte lampbrush chromosomes, may correspond to operons, or even clusters of operons, rather than to individual genes. Certainly the amount of DNA in a single band or loop is sufficient to represent a very large number of structural genes on the basis of evidence from procaryotic systems, provided all the DNA makes "sense" and is coding for specific polypeptides, a fact which is by no means established as yet. There remains the additional possibility that groups of two or more bands may act as single operons, as has been suggested by Lewis (*21*) for the bithorax mutants in Drosophila, and as may be true for certain puff sequences, such as those in the X chromosome of Drosophila discussed in the paper by Becker. Alternatively such systems may constitute still larger and more complex functional units, such as supra-operons, with special properties of which we are as yet unaware.

Pavan discussed the studies which he and his associates have performed with the salivary gland chromosomes of that remarkable organism, *Rhynchosciara angelae.* This material continues to pose many problems and also to provide many opportunities for further study. The evidence for the occurrence of extra replications of DNA in the chromosomal bands of this organism is of particular interest. In addition, we shall all anticipate further studies dealing with the recent evidence that infectious agents can induce marked general as well as specific cytologically detectable changes in the chromosomes of Rhynchosciara.

Some of the clearest evidence at the present time for an association between a particular visible structural region of a chromosome and a specific function is that for the nucleolus-organizing region in eucaryotes. As discussed in the paper by Perry, the evidence is now clear that this region is involved in coding for ribosomal nucleic acid. Morphological evidence and DNA-RNA hybridization experiments indicate that a significant fraction of a genome, of the order of ½ of 1 percent, is involved in such coding. Although this implies that hundreds or perphaps even thousands of cistrons, in the conventional sense, are included in this region, mechanisms apparently exist, as demonstrated by Brown and Guerdon in *Xenopus laevis* (*22*), by which the action of the entire region can be shut off—leading to the failure of nucleolus formation—in the absence of any cytologically detectable deletion in the nucleolus-organizing chromosome. This evidence strongly implies the existence of a regulatory mechanism that may be of a special operon type, possibly involving (at least in some instances, as shown by the very interesting observations reported by both Miller and Peacock) extra replications of a DNA segment to produce DNA rings which become free in the nucleus where they may transcribe ribosomal RNA.

In concluding, I would like to indicate once again, as will be obvious to those who have read the papers presented at this Symposium, that this summary has been, of necessity, incomplete. I have attempted primarily to emphasize the many basic similarities as well as the significant differences that characterize the organization and action of the genetic material in the two major groups of organisms—the procaryotes and eucaryotes. The papers presented in this Symposium provide impressive evidence concerning the remarkable advances that have occurred within the past few years in our knowledge of the structure and function of genetic systems. They also remind us of how much remains to be learned. I am confident that this Symposium will stimulate all of us to attack the remaining basic problems of genetics with increased insight and renewed enthusiasm.

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